REMARKS/ARGUMENTS

Reconsideration of the above-identified application is respectfully requested.

Election of Species

Initially, it is noted that the election of species has been withdrawn and a new one given, *to wit*:

(A) a telomere damage-inducing agent that is (A1) paclitaxel or a derivative thereof: (A2) platinum-based agents, such as cisplatin or carboplatin; or (A3) an agent other than (A1) or (A2);

(B) a telomerase inhibitory agent that is (B1) a nucleoside or nucleotide analog, such as, AZT or d4T; (B2) an antisense nucleic acid; or (B3) an agent that is neither (B1) or (B2),

wherein each patentably distinct species has a distinct telomere damage-inducing agent (A2, A2, or A3) and a distinct telomerase inhibitory agent (B1, B2, or B3).

Applicants hereby confirm their provisional election to prosecute telomere damage-inducing agent (A1) and telomerase inhibitory agent (B1), with traverse. These claims include: 1-24, 26-28, 33-35, 40-47, and newly added 90-92.

Summary of the Invention

The present invention is based on the discovery that paclitaxel treatment of a cancer causes telomere damage, thereby inducing telomerase activity and leading to resistance to paclitaxel treatment. Applicants also discovered that other cytotoxic agents, such as cisplatin, radiation, hyperthermia, and serum starvation, induce telomerase activity. Applicants further discovered that combining paclitaxel with a telomerase inhibitory agent, such as 3'-azido-deoxythymidine (AZT) or 2',3'-didehydro-3'deoxythymidine (d4T), results in synergy. These discoveries have led to the present invention for using combinations of an agent, such as paclitaxel, that causes telomere damage and an agent that inhibits telomerase, such as AZT, d4T, or antisense to the RNA component of human telomerase. Applicants further discovered that the AZT doses used to enhance the antitumor activity of paclitaxel are about 20-fold lower compared to the AZT doses used in the prior art. Similarly, the AZT concentrations needed to enhance the paclitaxel activity are at least several folds lower than the AZT concentrations needed to produce 50% inhibition of telomerase activity as shown in the prior art. Applicants further defined the AZT and d4T concentrations and the AZT doses that produce the greatest synergy with paclitaxel, whereas the prior art does not provide such enabling steps.

Claim Rejections

Claims 24, 26, 27, 45-47 have been amended to correct the typographical errors in the word "nucleotide" to the word "nucleoside", and to correct the typographical error of "dT4" to "d4T". No new matter is added by virtue of these claim amendments. Moreover, such claim amendments are ministerial in nature as they relate to inadvertent errors that are typographical in nature. Accordingly, Applicants assert that no claims have been narrowed with the meaning of *Festo* (*Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co.*, 535 US 722, 112 S.Ct. 1831, 152 L.Ed.2d 944, 62 USPQ2d 1705 (2002)). See also *Interactive Pictures Corp. v. Infinite Pictures Inc.*, Fed Cir., No. 01-1029, December 20, 2001 (addition of the words "transform calculation" was not a narrowing amendment because that addition did nothing more than make express what had been implicit in the claim as originally worded).

The remaining claim amendments will be discussed in connection with the rejections of the claims discussed below.

Claims 1-24, 26-28, 33-35, and 40-47 stand rejected to under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for the reduction of telomere length and treatment of cancer related to human breast MCF-7 cells, pharynx FaDu cells, prostate PC3 cells, and ovarian SKOV3 cells, does not provide enablement for inhibiting or reducing the growth of all types of cells or for treating all types of cancer. The Examiner cites the reasons being that undue experimentation is required and the unpredictability in the art. Initially, Applicants would remind the Examiner that working examples in the above-identified application include four tumor models, representing major types of human cancer, including FaDu, which is a head and neck cancer; PC3, which is a prostate cancer; SKOV-3, which is an ovarian cancer; and MCF-7, which is a breast cancer. Together, these cancer types account for about 50% of all adult human cancers. Furthermore, telomerase is expressed in 85-90% of human cancer cells, but not in normal somatic cells and telomerase is required for telomere maintenance, according to published information (see Declaration of Dr. Jessie L.-S. Au, Ph.D.). Based on this data and published information, it is Dr. Au's expert opinion that the invention is likely to also work with other cell types (*e.g.*, cancers) based on her and her co-worker's work to date. This data and expert opinion counter this claim rejection. Thus, its withdrawal respectfully is requested.

Claims 1-24, 28, 33-35, and 40-45 stand objected to under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for the reduction of telomere length and treatment of cancer related to human breast MCF-7 cells, pharynx FaDu cells, prostate PC3 cells, and ovarian SKOV3 cells, using a combination of paclitaxel and AZT or d4T, does not provide enablement for inhibiting or reducing the growth of all types of cells or for treating all

types of cancer, nor provide enablement for inhibiting or reducing the growth of a cell or for treating cancer using a combination of paclitaxel and a nucleoside or nucleotide analogue other than AZT or d4T. The Examiner states that not all nucleoside or nucleotide analogues are polymerase inhibitors. With respect to cell types treatable according to the precepts of the present invention, reference is made to the attached declaration of Dr. Au and the comments in the preceding paragraph. With respect to a nucleotide analog, reference is made to the application, wherein it is stated, *inter alia*:

> The term "nucleotide analog, or derivative thereof" refers to those art recognized modified nucleic acid bases that, typically, resemble a natural building block of DNA or RNA polymerization but have been modified to have an additional property such as, *e.g.*, the ability to inhibit a reverse transcriptase, *e.g.*, retroviral reverse transcriptases and telomerases.
>
> Application at page 11, ll. 32-36.

This definition certainly militates against the term being indefinite. Additional definitions of other analogs or derivatives and many other terms used in the application and claims are set forth in the definition section of the application at pp. 9-13. The data, Dr. Au's expert opinion, and the definitions in the application counter this claim rejection. Thus, its withdrawal respectfully is requested.

Claims 41 and 43 are rejected under 35 § U.S.C. 112, first paragraph, as not being enabling for identifying patients about to have cancer. The Examiner's attention respectfully is directed to the application, wherein it is stated, *inter alia*, that this term

> ...refers to a patient having been determined to have, or to be statistically likely to have, a cancer using various art recognized diagnostic or prognostic techniques including, *e.g.*, the PSA test, BRCA1 and/or BRCA2 genotyping, genetic profiling, *etc.* The term is also intended to include the mere knowing or receipt of any information (*e.g.*, a prognosis, diagnosis) indicating that the patient is having or about to have a cancer.
>
> Application at page 11, ll. 5-10

Thus, a definition of this term is set forth in the application. Indeed, the disputed term refers to patients that statistically are in a high-risk group to have cancer. Thus, it is not seen how this term is indefinite. Nevertheless, in order to materially advance prosecution of this application, the offending language has been removed from claims 41 and 43.

Claims 1-24, 26-28, 33-35, and 40-47 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject

matter which applicant regards as the invention due to the inclusion of the term, "telomere damage inducing agent". Initially, this term is defined in the specification:

> The term "telomere damage-inducing" refers to any measurable change to the end of a telomere when *e.g.*, compared to a control cell, chromosome, or nucleic acid and includes chromosomal fragmentation, telomere shortening, and the presence of DNA free ends.
>
> Application at page 12, ll. 23-26.

The term "telomere damage inducing agent" is enabled for an agent that causes damaged or shortened telomeres at a rapid onset, as described in Example 3; or prior to the initiation of apoptosis cascade, as described in Example 4. The term "telomere damage inducing agent" also refers to an agent that causes telomere damage, followed by a transient increase in telomerase activity, as described in Example 5. This term differs from "telomerase inhibitory agents", which are defined in the application as:

> The term "telomerase inhibitory agent" refers to an agent that inhibits (completely or partially) the activity of the enzyme telomerase.
>
> Application at page 12, ll. 27-29.

Thus, the term is not indefinite, as it has been defined in the application, and the term is different from "telomerase inhibitory agent", which similarly has been defined in the application. Nevertheless, in order to materially advance prosecution, the definitions in the Examples have been incorporated into claims 1 and 2. Again, no new matter is added by virtue of these claim amendments. Importantly, Applicants assert that no claims have been narrowed with the meaning of *Festo* (*Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co.*, 535 US 722, 112 S.Ct. 1831, 152 L.Ed.2d 944, 62 USPQ2d 1705 (2002)). See also *Interactive Pictures Corp. v. Infinite Pictures Inc.*, Fed Cir., No. 01-1029, December 20, 2001 (addition of the words "transform calculation" was not a narrowing amendment because that addition did nothing more than make express what had been implicit in the claim as originally worded).

102 (b) Claim Rejections Based on Gill

Claims 1-4, 8-10, 12-14, 16, 18, 20, 22-24, 26, 40, 42, and 44-46 stand rejected under 35 § U.S.C. 102(b) as being anticipated by Gill (U.S. Patent No. 5,756,537). Gill teaches that paclitaxel can be administered concurrently with AZT for the treatment of Kaposi's Sarcoma in patients with acquired immunodeficiency syndrome (AIDS).

This prior art in fact teaches that paclitaxel can be used to treat Kaposi's Sarcoma in AIDS patients who routinely received AZT and other reverse transcriptase inhibitors to manage the AIDS, but does not teach that adding AZT, d4T, or other reverse transcriptase inhibitors, through inhibition of telomerase, enhances the antitumor activity of paclitaxel. Hence, in the absence of the present invention, there is no motivation to use AZT, d4T or other reverse transcriptase inhibitors to enhance the telomere-directed effect of paclitaxel. Furthermore, the present invention enables the identification of an AZT treatment schedule to enhance the efficacy of paclitaxel, which cannot be found in the prior art, as the prior art does not provide guidance to finding such a treatment schedule. For example, the doses of AZT required for treatment of AIDS are higher compared to the AZT doses required to enhance the paclitaxel effect, as follows. The AZT doses used in AIDS patients are 100 mg every 4 hours given orally or 1 mg/kg given intravenously every 4 hours, given daily (PDR electronic library. Online version. Under Retrovir®). The present invention teaches that the synergy between paclitaxel and AZT is greatest when the dose ratio of AZT:paclitaxel is equal or less than 40:60 (see Example 8). Example 9 further shows that the intravenous AZT dose required to enhance the survival advantage of paclitaxel in tumor-bearing mice is 200 ng/hr/day. As the average weight of a mouse is about 20 g, this translates to 0.24 mg/kg/day. This dose is about 20-fold lower compared to the intravenous AZT dose of 5-6 mg/kg/day used to treat AIDS (PDR electronic library. Online version. Under Retrovir®). Claim 26 has been amended, and claims 91 and 92 have been added, to reflect the AZT doses and the ratios of AZT:paclitaxel concentrations discovered by the present invention.

§ 103(a) Rejection Based on Vande Woude in view of Merck Index

Claims 1-24, 26-28, 40, 42, and 44-47 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the Vande Woude (U.S. Patent No. 6,150,398), in view of The Merck Index, 1996, 7117,8958 and 1052. Vande Woude teaches that paclitaxel or a paclitaxel derivative can be used with an agent that effects the G1 or S phase of the cell division cycle. The Merck Index teaches that AZT and d4T are polymerase inhibitors, and that AZT has antiviral, antimetabolite, and antineoplastic activity. The Examiner proposes that polymerase inhibitors are often inhibitors of the G1 or S phase of cell division and, thereby, considers that the present invention is obvious based on combination of the prior art. However, Vande Woude did not define the AZT concentrations and doses that would affect the G1 or S phase of the cell division cycle. Chandrasekaren *et al.* showed that 50 to 200 micromolar AZT blocks cells in the G1/S phase (*Cancer Chemother Pharmacol* 35:489-495, 1995). The AZT dose required to produce 40 micromolar plasma concentration is 10 g/m2/day (Marchbanks *et al.*, *Pharmacotherapy*, 15:451-

457, 1995; Figure 1). Using a commonly accepted conversion factor of 37 kg/m2, the 10 g/m2/day dose equals about 270 mg/kg/day, which is about 1,000-fold higher than the 0.24 mg/kg/day AZT dose needed to produce synergy with paclitaxel. Furthermore, AZT is not known to have antiviral, antimetabolite, and antineoplastic activity at the low doses that enhance the antitumor activity of paclitaxel, as demonstrated in Example 9 in the present application (see the paragraph immediately below for detailed discussion). Hence, in the absence of the present invention, there is no motivation of combining paclitaxel and low dose AZT for the treatment of cancer or to use such low AZT doses.

The present invention on using d4T to enhance the efficacy of paclitaxel differs from the prior art in two respects. First, d4T does not always affect cells in the G1 or S phase. Li *et al.* shows that d4T arrests WiDr cells in the S phase but has no effect on MCF7 cells (Li *et al.*, *Anticancer Res* 17:21-28, 1997). This unpredictability in the art makes it unobvious to use d4T in combination with paclitaxel. Second, the present invention demonstrates that at least 20 micromolar d4T is needed to enhance the activity of paclitaxel, in view of Vande Woude. In comparison, the d4T concentration required to cause arrest of WiDr cells in the G1/S phase of the cell cycle is 10 micromolar (Li *et al.*). Accordingly, the present invention indicates the use of a 2-fold higher d4T concentration that the concentration that is needed to cause G1/S phase arrest. This d4T concentration requirement could not have been anticipated or made obvious by the Vande Woude publication. Claim 27 has been amended, and 90 has been added, to reflect the d4T concentration.

§ 103(a) Rejection Based on Melana in view of Merck Index

Claims 1-24, 26, 28, 40, 42, and 44-46 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the by Melana article, in view of the Merck Index. Melana teaches that AZT is effective in inhibiting the growth of four breast cancer cell lines and T4 cell leukemia, that AZT inhibits telomerase activity, and that AZT can be used, alone or in combination, as an anti-breast cancer agent. The Merck Index teaches that paclitaxel is a known antineoplastic for the treatment of breast of ovarian cancer.

The present invention is distinct from these earlier publications in several aspects. First, Melana teaches that AZT has antitumor activity at high concentrations; the 50% inhibitory concentrations of AZT were between 250 and 1,750 micromolar for the eight cell lines tested. In contrast, the present invention teaches using AZT to enhance the activity of paclitaxel, an effect that does not require AZT to have cytotoxic activity. Second, Melana teaches that inhibition of telomerase requires very high levels of AZT of between 500 to 2,000 micromolar AZT. In contrast, the present invention teaches using AZT at concentrations equal to or below 100

micromolar (see Example 8). Accordingly, Melana and the Merck Index do not teach using AZT together with paclitaxel, wherein the AZT concentration is equal to or below 100 micromolar. Third, although Melana suggests that AZT can be potentially used, alone or in combination, as an anti-breast cancer agent, the method of combining AZT with other drugs has not been explicitly disclosed. Several publications have described using AZT as antitumor agent, alone or in combination with other antitumor drugs (Marchbanks *et al.*, *Pharmacotherapy*, 15:451-457, 1995; Beitz et al., Cancer Investigation, 13:464-469, 1995; Posner et al, Cancer, 70:2929-2934, 1992; Posner *et al*, J. *Natl. Cancer Inst.*, 82:1710-1714, 1990). All these earlier studies used high doses of AZT, *i.e.*, between 2 to 20 g/m^2/day, and recommended a final dose of 7.5 g/m^2/day. Using the commonly accepted conversion factor of 37 kg/m^2, the 7.5 g/m^2/day dose is approximately 171 mg/kg/day. This AZT dose is about 680-fold higher than the 0.25 mg/kg/day AZT dose that enhances the efficacy of paclitaxel (see Example 9).

Fourth, Melana does not teach that the antitumor activity of AZT is due to telomerase inhibition. Melana states "the effect of AZT on other enzymes, such as DNA polymerase gamma and thymidine kinase, which are present in all cell types, cannot be ruled out" (p. 695, right column). Finally, telomerase is not considered a viable therapeutic target for cancer treatment (Huminiecki, *L. Acta Biochimica Polonica*, 43:531-538, 1996; Neidle and Kelland, *Anti-cancer Drug Design*, 14:341-347, 1999). Huminiecki states that "telomerase has not been usually considered to be a therapeutic target" (p.533, right column). Huminiecki further states "One can argue than even if such a (telomerase-targeting) therapy worked, its action would be very slow, and patients would probably die before telomeres of their malignant cells would shorten to a critical length" (p.534, left column). Neidle and Kelland state that "the emerging cellular biological properties of telomeres and telomerase as outlined above have caused some to question their validity as an anticancer target" (p. 342, first column). Hence, the use of telomerase inhibitor as described in the present invention is not obvioius based on the prior art. Further, the present invention, where AZT is used at doses of less than 170 mg/kg/day, cannot be obvious from the teaching of Melana, in view of the Merck Index. Claim 26 has been amended, and claims 91-92 have been added, to indicate the AZT dose or AZT concentrations identified by the present invention.

§ 103(a) Rejection Based on Melana in view of Merck Index and Pai

Claims 1-24, 26-28, 40, 42, and 44-47 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Melana in view of the Merck Index and Pai. Pai teaches that d4TTP inhibits telomerase in cell free extracts; 18% inhibition was achieved at 30 micromolar d4TTP. d4TTP is an intracellular metabolite of d4T. The prior art shows that incubation of human cells with 10

micromolar d4T yielded only 0.3 pmole of d4TTP per one million cells, indicating that only a very small percentage of d4T present in the extracellular fluid is converted to d4TTP its triphosphate metabolite (Zhu *et al.*, *Mol Pharmacol*, 40:838-845, 1991). There is no prior art to indicate the amount of intracellular d4TTP attained at 20-40 micromolar d4T in the extracellular matrix. A linear extrapolation based on the teaching of Zhu *et al.* and using the assumption that one million cells is approximately 1 microliter in volume, at least 1,000 micromolar extracellular d4T is required to yield 30 micromolar intracellular d4TTP. The 1,000 micromolar extracellular d4T concentration is at least 25-fold higher than the 20-40 micromolar extracellular d4T concentration used to improve the efficacy of paclitaxel, as is taught by the present invention. Accordingly, the present invention is not be rendered obvious by Melana, in view of Pai and the Merck Index. Claim 27 has been amended, and 90 has been added, to reflect the d4T concentration.

§ 103(a) Rejection Based on Gill in view of Merck Index

Claims 1-4, 7-28, 40, 42, and 44-47 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Gill, in view of the Merck Index. Gill teaches that paclitaxel can be administered concurrently with AZT to treat Kaposi Sacroma in AIDS patients, and that paclitaxel can be used with other antiretroviral agents that are used to treat AIDS. The Merck Index teaches that d4T is a reverse transcriptase inhibitor.

As discussed above, Gill does not teach using AZT to enhance the antitumor activity of paclitaxel and, therefore, does not teach how to find an AZT dose that can synergize with paclitaxel. In fact, Gill does not teach that any dose of AZT can synergize with paclitaxel. Hence, Gill does not render claims 1-4, 7-28, 33-35, and 40-47 unpatentable. The d4T doses used in AIDS patients are 60-80 mg per day (PDR electronic library. Online version. Under Zerit®). These doses would yield a maximum plasma concentration of about 4 micromolar (PDR electron library. Online version. Under Zerit®). The present invention teaches the synergy between paclitaxel and d4T where the d4T concentrations are at least 20 micromolar (see Example 8). This concentration is at least 5-fold higher compared to the concentration used to treat AIDS patients. Claims 26 and 27 have been amended, and claims 90-92 have been added, to reflect the distinguishing features of the present invention, regarding the AZT doses and concentrations, and the d4T concentrations.

Conclusion

In view of the amendments and remarks submitted herewith, allowance of the claims 1-24, 26-28, 33-35, 40-50, and 90-92 and passage to issue of this application respectfully are requested.

Respectfully submitted,

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ORIGINAL ARTICLE

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Synchronization of cells in the S phase of the cell cycle by 3′-azido-3′-deoxythymidine: implications for cell cytotoxicity

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Abstract The mechanism of synergy between 3′-azido-3′-deoxythymidine (AZT) and anticancer agents was investigated with emphasis on cell-cycle events. Exposure of exponentially growing WiDr human colon carcinoma cells to AZT resulted in synchronization of cells in the S phase of the cell cycle. Following treatment with AZT at 50 or 200 μ*M*, 62% ± 3% or 82% ± 4% of the cells were in the S phase as compared with 36% ± 2% in the control. Bromodeoxyuridine uptake studies revealed that the synchronized cells actively synthesized DNA. At concentrations of up to 200 μ*M*, AZT produced a cytostatic rather than cytotoxic effect as indicated by viability and cell growth measurements. At 200 μ*M*, AZT-induced synchronization was significant ($P = <0.001$) after 12 h of drug exposure, reached a maximum at 24 h, and reversed to baseline levels by 72 h even in the continued presence of the drug. This indicates that AZT-induced cytostasis is a transient and reversible effect. The cell-cycle events seen with AZT in WiDr cells were also observed in eight of nine human tumor cell lines tested. Isobologram analysis of WiDr cells preexposed to AZT for 24 h and then exposed to either AZT-5-fluorouracil or AZT-methotrexate for a further 72 h revealed synergy between AZT and the anticancer agents, indicating that AZT-induced synchronization may have therapeutic benefits.

Key Words AZT · S-phase cytostasis · cytotoxicity · DNA histograms · 5-FU · MTX · flow cytometry

Abbreviations *AZT* · 3′-Azido-3′-deoxythymidine · *5-FU*, 5-fluorouracil · *MTX* methotrexate · *BrdUrd* bromodeoxyuridine · *MTT* 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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Introduction

3′-Azido-3′-deoxythymidine (AZT, Retrovir) is active against human immunodeficiency virus [10,16] and is clinically used in patients with severe acquired immunodeficiency syndrome (AIDS)-related complex and AIDS [4,7,19]. AZT has been shown to act synergistically with the anticancer agents 5-fluorouracil (5-FU) and methotrexate (MTX) against human tumor cells [2,3,24,25]. The mechanism of action was postulated to be inhibition of thymidine kinase, since AZT is phosphorylated by this enzyme in cancer cells. As a result, AZT would be expected to block the synthesis of deoxythymidine triphosphate and should synergistically interact with inhibitors of deoxypyrimidine biosynthesis such as MTX and 5-FU, which are inhibitors of the thymidylate synthase cycle [24].

On the basis of studies in cisplatin-resistant cell lines, an additional role for AZT as an inhibitor of DNA repair and replication has also been postulated. Several studies led to the conclusion that cisplatin-resistant cells have an enhanced capacity to remove potentially lethal cisplatin-DNA adducts and resynthesize DNA in repair gaps [1,6,22]. The cisplatin-resistant cells overexpressed five enzymes (dihydrofolate reductase, thymidine kinase, thymidylate synthase, and DNA polymerases α and β) believed to be important for DNA replicative and repair synthesis [20]. These five enzymes are S-phase-specific enzymes and, moreover, DNA replicative and repair synthesis occurs in the S phase of the cell cycle. In earlier studies, inhibitors of DNA repair synthesis were shown to enhance the lethal effects of cisplatin [15,21]. The combination of AZT and cisplatin was 13 times more potent than an equivalent dose of cisplatin alone in cisplatin-resistant HCT-8 cells [20]. These observations led us to the

present investigation of changes in the cell-cycle events occurring in human tumor cells exposed to AZT.

In this study, we investigated AZT-induced cell-cycle perturbation and its relationship to cell growth and viability in human tumor cells. On the basis of these findings, we present a correlation between AZT-induced cell-cycle perturbation and synergistic cytotoxicity of combinations of AZT and 5-FU or AZT and MTX.

Materials and Methods

Chemicals and drugs

AZT and calcium leucovorin were supplied by Burroughs Wellcome Co. MTX and 5-FU were obtained from Sigma Chemical Co. Cell-culture medium was supplied by Gibco Laboratories, and fetal bovine serum was obtained from Hazelton Research Products, Inc. All other chemicals and reagents used were of analytical grade.

Cell culture

Human colon-carcinoma cell lines (WiDr, SW480, and SW620), a human melanoma cell line (A-375), a human pancreatic carcinoma cell line ($BxPC_3$), and a human breast carcinoma cell line (MCF-7) were obtained from the American Type Culture Collection (Rockville, Md.) The human leukemia cell line CCRF-CEM was provided by Dr. J. Bertino, Sloan Kettering Memorial Cancer Center (New York, NY.). The human colon carcinoma GC_3C_1 and its subline lacking thymidine kinase activity (GC_3/TK^-) were obtained from Dr.-J.-Houghton, St. Jude Hospital, (Memphis, Tenn.) [13]. The subline was selected with bromodeoxyuridine (Brdurd), was incapable growing in HAT medium, and was deficient in the cytosolic form of thymidine kinase. The WiDr, SW480, MCF-7, GC_3C_1, and GC_3C_1/TK^- cells were cultured in folate-free RPMI 1640 medium supplemented with 10 n*M* calcium leucovorin and 10% charcoal-dialyzed fetal bovine serum. All the remaining cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Growth inhibition (IC_{50} values) was determined using the MTT assay as described by Mossman [17]. Isobologram analysis was carried out as described previously [5].

DNA histogram and cell-cycle analysis

Exponentially growing human tumor cells were exposed to various concentrations of AZT and were harvested at different times using trypsin-ethylenediaminetetracetic acid (EDTA). The cells were fixed in ice-cold 70% ethanol (1 ml) and stored at 4°C for a minimum of 12 h but no longer than 1 week prior to further analysis. The cells were spun down at 1,000 *g* for 2 min and the supernatant was removed and discarded. The pellet containing cells (0.5–2-million) was stained for DNA with propidium iodide. In this procedure, the cell pellet was resuspended in 3.4-m*M* citrate buffer containing 0.01 *M* NaCl, 50 μg propidium iodide/ml, 0.6% NP-40, 37 μg RNAase/ml (75 Kunitz units/mg protein) at a pH of 7.6. Prior to analysis, the cells were passed through a 25-gauge needle and filtered through 40-μm mesh to remove any clumps. The cells were analyzed on a FACS STAR flow cytometer (Becton Dickinson FACS Systems) with excitation at 488 nm; the DNA fluorescence per cell was measured on a linear scale using a 630/22-nm bandpass filter. The list-mode data were collected for at least 20,000 particles using the Consort 30 program, and the DNA histogram data were analyzed using the MODFIT program (Verity Software). The determination of the distribution of cells in the G_1, S, and G_2 + M phases of the cell-cycle was based on a cell-size-gated population using defined markers for these parameters. There were at least duplicates for each analysis point, and the data given are mean values for the cell-cycle points.

BrdUrd incorporation as determined by flow cytometry

The cells exposed to AZT were further exposed to 8 μ*M* BrdUrd for 1 h at 37°C. The cells were spun at 1,000 *g* for 2 min and the supernatant was discarded. The cells were fixed with 70% ice-cold ethanol and stored at 4°C for a maximum of 1 week before further analysis. The pellet was washed with phosphate-buffered saline (PBS), treated with RNAse, acid-denatured, and treated with a fluorescein conjugate of anti-BrdUrd (Becton Dickinson Immuno Cytometry Systems) as described in the kit procedure. The DNA was then stained with 20 μ*g* propidium iodide/ml; the dual labeled cells were processed on a Becton Dickinson FACS STAR flow cytometer using excitation at 488-nm and measurement of log fluorescein isothiocyanate (FITC) incorporation per cell with a 530/30-nm bandpass filter. Controls for background fluorescence were carried out by elimination of addition of the primary antibody. The DNA fluorescence per cell was measured as described above. The two-color analysis was performed using the Consort 30 program.

Results

The results illustrated in Tables 1 and 2 confirm the findings obtained in other laboratories indicating that AZT interacts synergistically with both MTX and 5-FU. Treatment of WiDr cells for 24 h with AZT followed by further treatment for 72 h with the combination of AZT and MTX or AZT and 5-FU resulted in a synergistic inhibition of cell growth. AZT applied concurrently with or after the drugs exhibited additive effects. MTX and 5-FU are both cycle-specific antitumor drugs that are active in the S phase of the cell cycle. Moreover, although the IC_{50} of AZT in WiDr cells is high (approximately 100 μ*M*), this drug would also be expected to be active during the S phase. Thus, to determine whether the synergistic effects of AZT were related to changes in the cycling of the cells, we examined the effects of AZT on the cell cycle in WiDr cells.

Figure 1 illustrates changes in the cell-cycle distribution as determined by DNA staining with propidium iodide in WiDr cells treated with AZT at concentrations of 50 and 200 μ*M*. Treatment of the cells with AZT resulted in the accumulation of cells in the S phase. For the first 24 h of treatment with either concentration of AZT, there was a progressive accumulation of cells in the S phase with a corresponding decrease in the proportion of cells in G_1. Over the ensuing 48 h, there was cell division with an exit of cells from G_2 + M and reentry into G_1. Treatment of WiDr cells with AZT resulted in a significant increase ($P < 0.001$) in the percentage of cells in the S phase at

Table 1 Isobologram analysis of WiDr cells exposed to AZT and 5-FU[a]

Drugs (μM)	Validity	
	Observed	Predicted
AZT (100)	50.0	50
AZT (90) + 5-FU (0.18)	49.3	50
AZT (80) + 5-FU (0.36)	38.8	50
AZT (70) + 5-FU (0.54)	33.0	50
AZT (60) + 5-FU (0.72)	34.8	50
AZT (50) + 5-FU (0.9)	36.6	50
AZT (40) + 5-FU (1.08)	35.2	50
AZT (30) + 5-FU (1.26)	38.1	50
AZT (20) + 5-FU (1.44)	38.8	50
AZT (10) + 5-FU (1.62)	44.5	50
5-FU (1.8)	50.0	50

[a] A total of 2×10^3 cells were seeded in a 96-well microtiter plate, each well containing a volume of 150 μl. AZT was added 2 h later and 5-FU was added 24 h after AZT; the final volume was 300 μl. Under the experimental conditions the IC_{50} for 5-FU was 1.8 μM and that for AZT, 100 μM. The cells were incubated and processed, and viability was determined as described in Materials and methods. The results are mean values for a minimum of six data points

Table 2 Isobologram analysis of WiDr cells exposed to AZT and MTX[a]

Drugs (μM)	Validity	
	Observed	Predicted
AZT (100)	50.0	50
AZT (90) + MTX (0.0003)	45.5	50
AZT (80) + MTX (0.0006)	43.2	50
AZT (70) + MTX (0.0009)	40.8	50
AZT (60) + MTX (0.0012)	39.7	50
AZT (50) + MTX (0.0015)	34.8	50
AZT (40) + MTX (0.0018)	38.0	50
AZT (30) + MTX (0.0021)	40.6	50
AZT (20) + MTX (0.0024)	42.9	50
AZT (10) + MTX (0.0027)	46.1	50
MTX (0.003)	50.0	50

[a] Under the experimental conditions the IC_{50} for MTX was 0.003 μM and that for AZT, 100 μM. The cells were seeded and processed, and viability was determined as described in Table 2. AZT was added 24 h prior to MTX. The results are mean values for a minimum of six data points.

12 h after drug exposure, which at 24 h reached a maximum of 62% ± 3% and 82% ± 4% for AZT concentrations of 50 and 200 μM, respectively (Table 3). The percentage of cells in the S phase returned to baseline levels by 72 h, even in the continued presence of the drug. In controls, 36% + 2% of the cells accumulated in the S phase at 24 h.

Consistent with the cell-cycle effects, WiDr cells exposed to 50 μM AZT exhibited an increased division time during the first 24 h followed by growth resumption comparable with control values during the next 48 h. In the case of 200 μM AZT, there was no growth

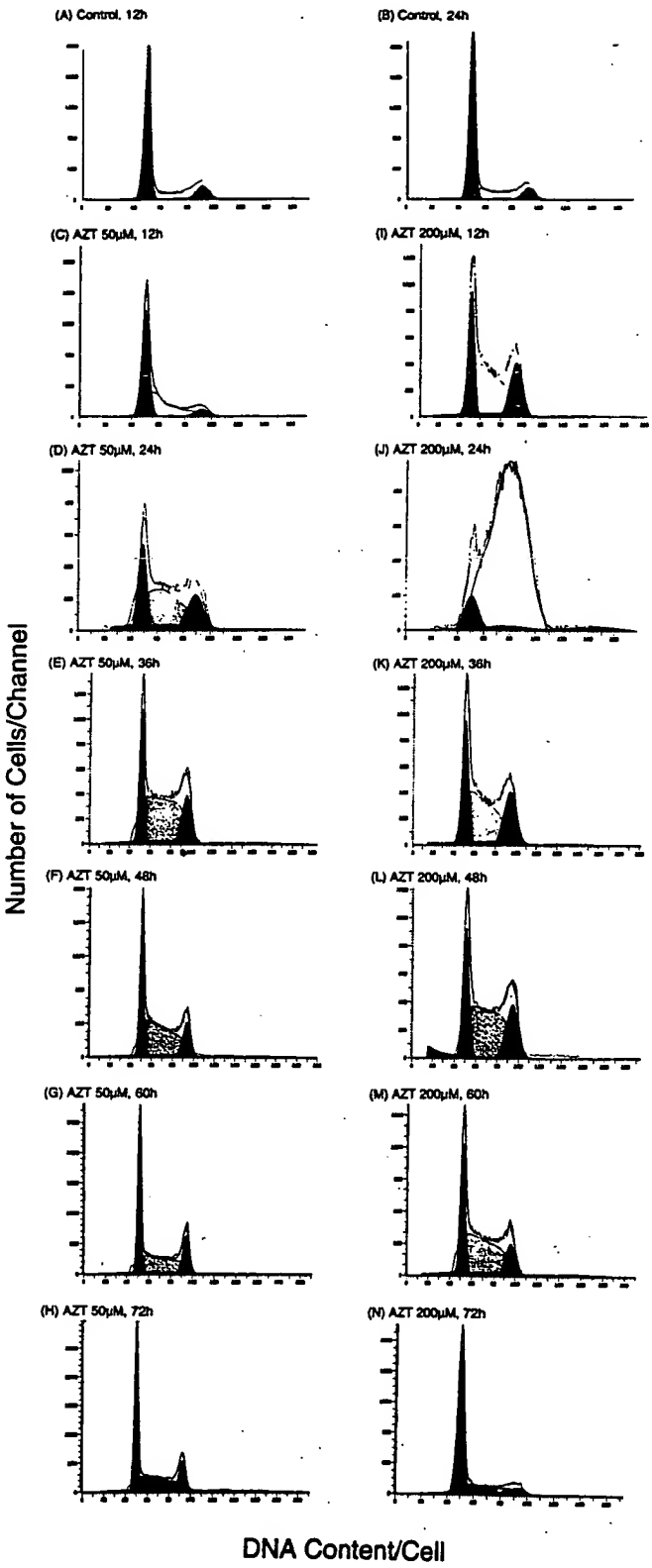

Fig. 1A–N DNA histograms of propidium iodide-stained WiDr cells. **A** controls at 12 h. **B** controls at 24 h. **C–H** Cells treated with 50 μM AZT for 12–72 h and **I–N** cells treated with 200 μM AZT for 12–72 h as described in Materials and methods. The peak visible at channel number 50 is G_1 and that visible at channel number 90–95 is G_2/M

Table 3 DNA historgrams of propidium iodide-stained WiDr cells exposed to AZT[a]

Drug	% Cells in		
	G_1	S	G_2 + M
Control, 12 h	46	36	18
AZT, 50 μM, 12 h	37	55	8
AZT, 200 μM, 12 h	34	61	5
control, 24 h	47	36	17
AZT, 50 μM, 24 h	30	62	8
AZT, 200 μM	14	82	4
Control, 36 h	40	40	20
AZT, 50 μM, 36 h	26	53	21
AZT, 200 μM, 36 h	17	61	22
Control, 48 h	44	37	19
AZT, 50 μM, 48 h	25	54	21
AZT 200 μM, 48 h	20	62	18
Control, 60 h	48	37	15
AZT 50 μM, 60 h	35	48	17
AZT 200 μM, 60 h	37	48	15
Control, 72 h	48	36	16
AZT 50 μM, 72 h	41	44	15
AZT 200 μM, 72 h	40	43	17

[a] The numbers were generated using MODFIT analysis

in the first 24 h, which was consistent with an almost total block of the cells in the S phase (Fig. 1). Growth retardation occurred during the next 24 h, which was followed by a return to control growth rates within 72 h, even in the continued presence of the drug. Trypan blue dye exclusion showed a viability of 95% at all time points (Table 4). The AZT-induced cell-cycle perturbations were time- and concentration-dependent over a concentration range of 3–200 μM. There was no cell-cycle perturbation effect during the first 5 h of AZT exposure. AZT-induced cell-cycle perturbations were maximal at 12–24 h after drug addition and then returned to baseline levels, even in the presence of the drug. The cell-cycle effects observed with AZT in WiDr cells were also observed in colon carcinomas SW480 and SW620, MCF-7 breast carcinoma, BxPC3 pancreatic carcinoma, A375 melanoma, and CCRF-CEM leukemia (Table 5). The results illustrated in Table 6 indicate that phosphorylation of AZT is required for synchronization of cells. GC_3TK^- cells, which lack the enzyme thymidine kinase and, thus, the ability to phosphorylate AZT to the monophosphate, do not exhibit synchronization in the presence of AZT. In contrast, treatment of the parent line from which this clone was derived (GC_3C_1) with 200 μM AZT led to the accumulation of approximately 90% of the cells in the S phase at 24 h.

BrdUrd uptake studies using a fluorescein conjugate of an antibody to BrdUrd (Table 7) showed that the cells blocked in the S phase by AZT actively synthesized DNA and that less than 3% of the AZT-treated cells were in the dormant SO stage. This further confirms that exposure of exponentially growing WiDr cells to AZT at concentrations of up to 200 μM for 72 h is not cytotoxic.

Discussion

In this report, we describe a novel mechanism by which AZT can produce a synergistic interaction with S-phase-specific anticancer agents. AZT caused a time and concentration-dependent synchronization of human tumor cells in the S phase of the cell cycle. This synchronization was dose-dependent, transient, reversible, and cytostatic in nature. AZT at 200 μM produced an almost total arrest of cells in the S phase of the cell cycle, whereas at lower concentrations, transit through the cell cycle was slowed significantly, though not completely. Synchronization parallels and may be related to the decreases in deoxynucleotides observed following treatment of cells with AZT [8,9]. These studies [8,9] showed that treatment with 200 μM AZT resulted in decreased levels of deoxythymidine triphosphate (dTTP) and deoxyguanosine triphosphate (dGTP), which returned to near normal levels by 24 h. When cells are treated with AZT, the transient decrease in dTTP occurs at the same time as an increase in the levels of AZT and its phosphorylated derivatives, including AZT triphosphate (AZTTP). Since AZT and thymidine compete for the same enzymes (i.e., thymidine kinase, thymidylate kinase, and DNA polymerase), decreased levels of dTTP would allow for the increased incorporation of AZTTP into DNA. As dTTP levels again increase, there would be increased incorporation of dTTP into DNA relative to AZTTP due to the more favorable kinetic parameters of thymidine and dTTP for the synthetic enzymes. This

Table 4 Growth rates of WiDr cells proposed to AZT

Drug	Doubling time (h)				% Viability[a]		
	0–24	24–48	48–72	0–72	24 h	48 h	72 h
Control	19.6	21.6	23.6	21.7	100	100	98
AZT, 50 μM	90.6	19.5	23.6	27.7	100	100	97
AZT, 200 μM	NG	41.4	22.0	48.0	98	100	98

[a] Viability was assessed using trypan blue dye exclusion
(*NG*, no growth)

Table 5 Cell-cycle distribution in human tumor cells exposed to AZT[a]

	% Cells in		
	G_1	S	G_2 + M
CCRF-CEM:			
Control, 12 h	16	54	30
AZT, 50 μM, 12 h	30	55	15
AZT, 200 μM, 12 h	0	65	35
Control, 24 h	40	45	15
AZT, 50 μM, 24 h	27	68	5
AZT, 200 μM, 24 h	0	89	11
SW-480:			
Control, 12 h	40	46	14
AZT, 50 μM, 12 h	25	70	5
AZT, 200 μM, 12 h	13	87	0
Control, 24 h	39	48	13
AZT, 50 μM, 24 h	34	52	14
AZT, 200 μM, 24 h	22	69	9
SW-620:			
Control, 12 h	40	45	15
AZT, 50 μM, 12 h	27	68	5
AZT, 200 μM, 12 h	23	73	4
Control, 24 h	38	45	17
AZT, 50 μM, 24 h	20	65	15
AZT, 200 μM, 24 h	12	88	0
MCF-7:			
Control, 12 h	56	22	22
AZT, 50 μM, 12 h	46	47	7
AZT, 200 μM, 12 h	53	39	8
Control, 24 h	52	28	20
AZT, 50 μM, 24 hz	41	57	2
AZT, 200 μM, 24 h	49	49	2
BxPC3:			
Control, 12 h	63	16	21
AZT, 50 μM, 12 h	52	38	10
AZT, 200 μM, 12 h	55	40	5
Control, 24 h	44	34	22
AZT, 50 μM, 12 h	16	78	6
AZT, 200 μM, 12 h	7	93	0
A375:			
Control, 12 h	15	55	30
AZT, 50 μM, 12 h	2	74	24
AZT, 200 μM, 12 h	0	79	21
Control, 24 h	17	54	29
AZT, 50 μM, 24 h	11	64	25
AZT, 200 μM, 24 h	0	94	6

[a] Cells exposed to AZT were fixed in ice-cold 70% ethanol and kept in a refrigerator for a minimum of 12 h (but no more than a week) until analysis by flow cytometry as described in Materials and methods

Table 6 Role of thymidine kinase in synchronization of cells by AZT

	% Cells in		
Cell/treatment	G_1	S	G_2 + M
GC3C1:			
Control, 12 h	36	42	22
AZT, 50 μM, 12 h	37	40	23
AZT, 200 μM, 12 h	43	40	17
Control, 24 h	47	32	21
AZT, 50 μM, 24 h	11	78	11
AZT, 200 μM, 24 h	6	91	3
GC3/TK$^-$:			
Control, 12 h	49	33	18
AZT, 50 μM, 12 h	52	25	23
AZT, 200 μM, 12 h	52	24	24
Control, 24 h	49	32	19
AZT, 50 μM, 24 h	48	27	25
AZT, 200 μM, 24 h	45	34	21

Table 7 BrdUrd uptake studies in WiDr cells exposed to AZT

	% Cells in				
	G_1	S			G_2 + M
		ES	LS	SO	
Control, 24 h	27		57:		16
		19	35	3	
AZT, 50 μM, 24 h	18		66:		16
		16	45	5	
AZT, 200 μM, 24 h	10		84:		6
		30	50	4	
Control, 72 h	31		51:		18
		18	30	3	
AZT, 50 μM, 72 h	27		52:		21
		13	39	3	
AZT, 200 μM, 72 h	22		60:		18
		14	46	0	

return corresponded to the time in these studies at which the cell-cycle distribution began to reverse toward normal.

The ability of AZT to induce synchronization appeared to be dependent on phosphorylation of the nucleoside. Treatment of GC3TK$^-$, a cell line deficient in thymidine kinase, the enzyme responsible for the phosphorylation of AZT, had no effect on the cell-cycle distribution in this cell line. Moreover, preliminary studies have indicated that the extent of the S phase block correlates with the amount of AZT incorporated into DNA (D.Duch, unpublished data). It had previously been shown [9] that AZT could synchronize the leukemic cell line CCRF-CEM in the S phase of the cell cycle. The results of this study show that AZT can also effectively synchronize cells derived from solid tumors.

Recently, Keyomarsi et al. [14] described an effective procedure for the synchronization of tumor cells using lovastatin. This procedure arrested mammalian cells in the G_1 phase of the cell cycle but required a second agent, mevalonic acid, to reverse the synchrony. In contrast, AZT-induced synchronization is reversible without addition of a second agent, even in the

continued presence of the drug. AZT treatment did not result in cell killing as determined by viability and cell growth measurements. BrdUrd uptake studies revealed that the arrested cells actively synthesized DNA, indicating the cytostatic nature of the AZT-induced cell-cycle effect. One approach to optimization of therapy is based on observations that the cell-cycle-traversing properties of malignant tumors and therapy-limiting host tissues are often distinctly different and that a variety of anticancer agents exist that kill or block cells only in limited parts of the cell cycle [11]. Cytokinetic strategies are based on observations that the tumor and/or normal cells can be partially synchronized during therapy and that the administration of cell-cycle-specific cytotoxic agents can be timed to maximize tumor-cell killing and/or to minimize normal-cell killing. These results suggest that pretreatment with AZT may modulate the tumor cells favorably to increase the therapeutic index of the anticancer agent.

Pretreatment of WiDr cells for 24 h with AZT followed by the combination of AZT and 5-FU or AZT and MTX resulted in synergistic cell-growth inhibition. Both 5-FU and MTX are S-phase-specific anticancer agents. The synergistic cytotoxicity seen in this study may have been due to synchronization of cells in the S phase of the cell cycle induced by AZT due to pretreatment followed by lysis of those cells in the S phase by the S-phase-specific cytotoxic agents 5-FU and MTX. Other mechanisms for the mode of action of AZT have been proposed. Weber et al. [24,25] proposed that through its inhibition of thymidine salvage, AZT synergistically enhanced the antitumor effects of MTX and 5-FU, both inhibitors of de novo thymidylate biosynthesis. Although inefficient as a substrate for cellular DNA polymerases, AZT can be incorporated into DNA and, when incorporated, acts as a chain terminator [18,23]. AZT that has been incorporated into DNA can be removed by a 3′-exonuclease activity, although the repair process may be saturated at high levels of AZT. Harrington et al. [12] have shown that AZT monophosphate is an inhibitor of 3′exonuclease purified from two different human cell lines and that the high levels of AZT monophosphate present following AZT treatment may be sufficient to inhibit the exonuclease activity and, hence, DNA repair. Indeed, Scanlon et al. [20,21] have proposed that AZT potentiates the antitumor activity of cisplatin through its effects on DNA repair.

In summary, we report a novel mechanism of synchronization of human tumor cells by AZT leading to synergistic interaction with S-phase-specific agents. This warrants further investigation in vitro and in vivo.

References

1. Ahnstrom G (1989) Inhibition of DNA strand break rejoining in ultraviolet-irradiated human cells: comparision of aphidicolin and cytosine arabinoside. Biochim Biophys Acta 1007: 357–358
2. Brunetti I, Darnowski JW, Falcone A, Johnson KA, Calabresi P (1989) Azidothymidine enhances fluorouracil and methotrexate antitumor and therapeutic activity. Proc Am Assoc Cancer Res 30: 595
3. Brunetti I, Falcone A, Calabresi P, Goulette FA, Darnowski JW (1990) 5-Fluorouracil enhances azidothymidine cytotoxicity: In vitro, in vivo, and biochemical studies. Cancer Res 50: 4026–4031
4. Chaisson RE, Allain J-P, Leuther M, Volberding PA (1986) Significant changes in HIV antigen level in the serum of patients treated with azidothymidine. N Engl J Med 315: 1610–1611
5. Chou T, Talay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22: 27–55
6. Eastman A, Schulte N (1988) Enhanced DNA repair as a mechanism of resistance to *cis*-diamminedichloroplatinum (II). Biochemistry 27: 4730–4734
7. Fischl MA, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Schooley RT, Jackson GG, Durack DT, King D, the AZT Colloborative Working Group (1987) The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex: a double-blind, placebo-controlled trial. N Engl J Med 317: 185–191
8. Frick LW, Nelson DJ, St Clair MH, Furman PA, Krenitsky TA (1988) Effect of 3′-azido-3′-deoxythymidine on the deoxynucleotide triphosphate pools of cultured human cells. Biochem Biophys Res Commun 154: 124–129
9. Fridland A, Connelly MC, Ashmun R (1990) Relationship of deoxynucleotide changes to inhibition of DNA synthesis induced by the antiretroviral agent 3′-azido-3′-deoxythymidine and release of its monophosphate by human lymphoid cells (CCRF-CEM). Mol Pharmacol 37: 665–670
10. Furman PA, Fyfe JA, St Clair MH,Weinhold K, Rideout JL, Freeman GA, Nusinoff-Lehrmann S, Bolognesi DP, Broder S, Mitsuya H, Barry DW (1986) 3′-Azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc Natl Acad Sci USA 83: 8333–8337
11. Gray JW, Pallavicini MG (1982) Ara-C scheduling: theoretical and experimental considerations. Med Pediatr Oncol 10 [suppl 1]: 93–108
12. Harrington JA, Reardon JE, Spector T (1993) 3′-Azido-3′-deoxythymidine (AZT) monophosphate: an inhibitor of exonucleolytic repair of AZT-terminated DNA. Antimicrob Agents Chemother 37: 918–920
13. Houghton PJ, Houghton JA, Germain G, Torrance PM (1987) Development and characterization of a human colon adenocarcinoma xenograft deficient in thymidine salvage. Cancer Res 47: 2117–2122
14. Keyomarsi K, Sandoval L, Band V, Pardee AB (1991) Synchronization of tumor and normal cells from G_1 to multiple cell cycles by lovastatin. Cancer Res 51: 3602–3609
15. Lai G-M, Ozols RF, Smyth JF, Young RC, Hamilton TC (1988) Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. Biochem Pharmacol 37: 4597–4600
16. Mitsuya H,Weinhold KJ, Furman PA, St Clair MH, Nusinoff-Lehrman S, Gallo RC, Bolognesi D, Barry DW, Broder S (1985) 3′-Azido-3′-deoxythymidine (BW A509 U): an antiviral agent that inhibits the infectivity and cytopathic effect on human T-lymphotropic virus type 111/lymphadenopathy-associated virus in vitro. Proc Natl Acad Sci USA 82: 7096–7100
17. Mossmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63

18. Nickel W, Austermann S, Bialek G, Grosse F (1992) Interactions of azidothymidine triphosphate with the cellular DNA polymerases α, δ, and ε and with DNA primase. J Biol Chem 267: 848–854
19. Richman DD, Fischl MA, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Hirsch MS, Jackson GG, Durack DT, Nusinoff-Lehrman S, the AZT Colloborative Working Group (1978) the toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. N Engl J Med 317: 192–197
20. Scanlon KJ, Kashani-Sabet M, Sowers LC (1989) Overexpression of DNA replication and repair enzymes in *cis*platin-resistant human colon carcinoma HCT8 cells and circumvention by azidothymidine. Cancer Commun 1: 269–275
21. Scanlon KJ, Funato T, Pezeshki B, Tone T, Sowers LC (1990) Potentiation of azidothymidine cytotoxicity in *cis*platin-resistant human ovarian carcinoma cells. Cancer Commun 2: 339–343
22. Swinnen LJ, Barnes DM, Fisher SG, Albain KS, Fisher RI, Erickson LC (1989) 1-β-D-Arabinofuranosylcytosine and hydroxyurea production of cytotoxic synergy with *cis*-diamminedichloroplatinum(II) and modification of platinum-induced DNA interstrand cross-linking. Cancer Res 49: 1383–1389
23. Vazquez-Padua MA, Starnes MC, Cheng Y-C (1990) Incorporation of 3′-azido-3′-deoxythymidine into cellular DNA and its removal in a human leukemic cell line. Cancer Comm 2: 55–62
24. Weber G, Ichikawa S, Nagai M, Natsumeda Y (1990) Azidothymidine inhibition of thymidine kinase and synergistic cytotoxicity with methotrexate and 5-fluorouracil in rat hepatoma and human colon cancer cells. Cancer Commun 2: 129–133
25. Weber G, Nagai M, Prajda N, Nakamura H, Szekeres T, Olah E (1991) AZT: a biochemical response modifier of methotrexate and 5-fluorouracil cytotoxicity in human ovarian and pancreatic carcinoma cells. Cancer Commun 3: 127–132

CLINICAL RESEARCH ARTICLES

Pharmacokinetics and Pharmacodynamics of High-Dose Zidovudine Administered as a Continuous Infusion in Patients With Cancer

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Study Objective. To investigate the pharmacokinetics and pharmacodynamics of high-dose intravenous zidovudine (ZDV).

Design. Phase 1, dose-escalating, unblinded study in patients with cancer.

Setting. A university-affiliated cancer treatment center.

Patients. Fourteen patients (6 women) with solid tumors that were unresponsive to standard therapy received 31 courses of therapy.

Interventions. Intravenous ZDV was administered in doses of 2, 3, 4, 5.5, 7, 8.5, 10, 12, 15, or 20 g/m^2/day as a continuous infusion over 48 hours. Patients also received fluorouracil plus leucovorin for 24 hours before the start of and during the ZDV infusion. If no dose-limiting toxicities were encountered, subsequent doses were escalated. Blood samples were collected at 24 and 48 hours after the start of the infusion, and hourly for 4 hours after stopping the infusion. Urine was collected in five patients during the infusion and for 24 hours after stopping it. Blood for measuring peripheral white blood cells was collected before and at the end of the infusion in seven patients to measure DNA chain breaks due to incorporation of ZDV.

Measurements and Main Results. Zidovudine was measured in plasma by high-performance liquid chromatography and in urine fluorescence polarization immunoassay. Its incorporation into DNA was measured by determining DNA strand breakage in peripheral white blood cells using fluorescence analysis. Pharmacokinetic models were fit to plasma ZDV concentrations using extended least squares regression. Short-term high-dose ZDV was generally well tolerated, with adverse effects related to large amounts of free water administered during the infusion. The mean (SD) ZDV pharmacokinetic values were total clearance 1.44 (1.09) L/hr/kg, volume of distribution 2.72 (2.97) L/kg, and half-life 1.2 (0.6) hours. There was considerable interpatient variability in total drug clearance. Although ZDV exposure increased proportionately with increasing dose, two of three patients receiving the highest dose (20 g/m^2/day) had markedly low total drug clearances. The relation between the percentage of abnormal DNA in peripheral white blood cells and zidovudine area under the plasma ZDV versus time curve was described by the E_{max} pharmacodynamic model.

Conclusions. The pharmacokinetics of high-dose ZDV administered by continuous infusion to patients with cancer are similar to those reported

with lower doses in patients with infection due to the human immunodeficiency virus. Further study of potential nonlinear pharmacokinetic behavior at doses above 20 g/m²/day is necessary. The high between-patient variability in ZDV clearance results in variable levels of exposure in vivo, and indicates the need for concentration- or effect-controlled study designs in the further evaluation of the agent's antineoplastic effects.
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Zidovudine (ZDV) is a pyrimidine nucleoside that was initially developed as an antineoplastic agent. Due to its limited antitumor activity as a single agent, this use was abandoned. The drug was ultimately shown to have antiretroviral activity.[1–3]

Recent studies demonstrated ZDV's antineoplastic efficacy when combined with agents that inhibit thymidylate synthesis, such as 5-fluorouracil (5-FU) and methotrexate.[4–6] Inhibition of de novo pyrimidine biosynthesis results in thymidine salvage pathways becoming the chief source of thymidylate for DNA synthesis. Thymidine salvage is mediated primarily by the enzyme thymidine kinase; this enzyme has a high affinity for ZDV and results in anabolic phosphorylation of the compound to the triphosphate metabolite. In the presence of inhibitors of de novo pyrimidine biosynthesis, ZDV triphosphate competes with thymidylate from salvage pathways during DNA synthesis; ZDV is incorporated into DNA. This interrupts DNA synthesis by terminating DNA chains. Combinations of ZDV plus inhibitors of de novo thymidylate synthesis display synergistic antitumor effects in human colon tumor cell lines in vitro, and in vivo in athymic (nude) mice bearing tumor xenographs, without increased toxicity[5–7]; however, the antitumor effect of the combination occurs at high concentrations of ZDV. This need for high concentrations requires administration of doses higher than those previously given in the standard treatment of human immunodeficiency virus (HIV) infection.

We assessed the pharmacokinetics of high-dose ZDV in patients with advanced malignancies receiving combination therapy of ZDV, 5-FU, and leucovorin. Details concerning clinical response and drug tolerance with this regimen are published elsewhere.[8]

Methods

Patients

Patients with histologically documented malignancy with no standard alternative therapy were eligible for enrollment if they met the following criteria: serum creatinine 1.6 mg/dl or less; liver enzymes less than or equal to 10 times normal; expected survival of 8 weeks or more; and Eastern Cooperative Oncology Group (ECOG) performance status less than 3 (bedridden ≤ 50% to fully ambulatory). The protocol was approved by a human research review committee, and informed consent was obtained for all patients.

Study Design

All patients were admitted to the hospital for treatment and received 5-FU 800 mg/m²/day as a continuous infusion, plus leucovorin 50 mg every 6 hours for 72 hours. Twenty-four hours after the start of 5-FU-leucovorin, a continuous infusion of ZDV (Burroughs Wellcome Co., Research Triangle Park, NC) was started and continued for 48 hours. The doses of ZDV were 2, 3, 4, 5.5, 7, 8.5, 10, 12, 15, and 20 g/m²/day. At least two patients were treated at each dose level, and in any patient who experienced no dose-limiting toxicities, subsequent courses were escalated. Patients were eligible for retreatment at a higher dose if the previous course was tolerated.

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Sample Collection

An indwelling venous catheter was placed before ZDV therapy, and blood samples were obtained at 24 and 48 hours after the start of the 48-hour infusion. Additional samples were collected hourly for 4 hours after the conclusion of the infusion. Plasma was harvested by centrifugation and stored at -70°C until assay.

In a subset of five patients, all urine was collected and pooled during 0–24, 24–48, 48–52, 52–56, and 57–72 hours after the start of ZDV infusion. Urine volume was recorded and a 5-ml aliquot was frozen at -70°C for assay of ZDV and ZDV glucuronide contents.

Zidovudine Assay

Concentrations of ZDV in plasma were quantified by high-performance liquid chromatography (HPLC) using previously reported methods.[9] Ice-cold 15% trichloroacetic acid 200 µl was added to 100 µl of plasma, mixed, and centrifuged. After centrifugation, the supernatant was neutralized by extraction with an equal volume of 1N trioctylamine in freon. This mixture was vortexed and the ZDV content of the aqueous material was quantified by the injection of 50 µl onto a C-18 analytic column (4.6 mm x 25.0 cm; Microsorb; Rainin, Woburn, MA) of the HPLC system. The column temperature was maintained at 20°C. Zidovudine was eluted at 1 ml/minute with a mobile phase of 30% methanol in water, and monitored at 254 nM using a fixed-wavelength detector. Under these conditions, the recovery of ZDV from plasma was 95% or greater. The retention time for ZDV was 11.5 minutes, and ultraviolet detection was linear over a concentration of 0.5–100 µM.

Concentrations of ZDV and ZDV glucuronide in urine were determined using fluorescence polarization immunoassay (FPIA; Sigma Diagnostics, St. Louis, MO) on an Abbott TDx system (Abbott Laboratories, Abbott Park, IL). All samples were diluted by at least 1:20 in pooled human serum to bring concentrations into the range of the assay. For measuring ZDV glucuronide concentrations in urine, each urine sample was divided and one aliquot was assayed directly for ZDV content. The second aliquot was combined with 1250 U β-glucuronidase (Sigma Diagnostics) and incubated at 37°C for 60 minutes to release ZDV from the glucuronide conjugate; this aliquot was then assayed for total ZDV content. The molar difference in concentrations between aliquots was determined to be the ZDV glucuronide concentration. Validation studies demonstrated that the recovery of ZDV from ZDV glucuronide using this method was approximately 100% (data not shown).

The within- and between-day variability of three seeded control samples of 490, 160, and 100 ng/ml ranged between 1.7% and 8% (coefficient of variation x 100%). At least one control was assayed with a set of 12 unknown urine samples. Since each sample underwent dilution, and the lower limit of quantification was 0.7 ng/ml, the lowest quantifiable concentration of ZDV glucuronide in urine was 14 ng/ml.

Pharmacokinetic Analysis

A one- or two-compartment pharmacokinetic model was fitted to plasma ZDV data using extended least squares regression.[10] The variance model was Y^{PWR}, where Y is the estimated plasma ZDV concentration, and PWR is a value constrained between 0 and 3. Selection of the pharmacokinetic model was guided by inspection of weighted residual plots for fitted data, and the Schwartz criterion, which invokes the rule of parsimony in the model discrimination process through consideration of the log likelihood of the model with the number of parameters used in the model.[11] The area under the plasma ZDV versus time curve (AUC) was calculated by dividing the dose by the estimate of clearance returned form the model. The elimination half-life was calculated by $0.693/\lambda_z$, where λ_z is the slope of the terminal elimination phase returned from the model fit. The volume of distribution was calculated as Dose/(AUC • λ_z). Renal clearance was calculated by the equation $Clr = Ae(t_1\text{-}t_2)/AUC(t_1\text{-}t_2)$, where Ae is the amount of drug excreted in the urine, and AUC is the corresponding plasma AUC during time t_1 to t_2. Nonrenal clearance was calculated by the difference between total clearance and renal clearance during the 48-hour infusion. Creatinine clearance was calculated by the method of Cockcroft and Gault.[12] The relation between the administered ZDV dose and AUC was examined by linear regression, and the outlier test was performed to determine if higher doses resulted in a disproportional increase in AUC.[13]

Pharmacodynamic Analysis

Zidovudine's incorporation into DNA was measured by determining DNA strand breakage using a fluorescence analysis of DNA structural

Table 1. Characteristics of 14 Patients

Characteristic	Value
Median (range) age (yrs)	64 (42–78)
No. (%) women	6 (43)
Mean (± SD) body weight (kg)	68.3 (12.7)
Tumor site, no. (%) patients	
Colon	10 (72)
Lung, head/neck	3 (21)
Other	1 (7)
Mean (± SD) calculated creatinine clearance (ml/min)	66.2 (24.1)
No. (%) patients who received 5-FU previously	7 (50)
No. courses of ZDV	
2	3
3	4
4	3
5.5	3
7	2
8.5	4
10	5
12	2
15	2
20	3

changes modified for whole blood cell populations as described elsewhere.[8,9] Blood was collected in tubes containing EDTA for evaluating DNA strand breaks in peripheral white cells before and at the conclusion of the ZDV infusion. The results were expressed as percentage of abnormal DNA compared with daily controls from a healthy volunteer. The relation between drug exposure and DNA strand breaks was examined using the E_{max} and sigmoid E_{max} models.[14]

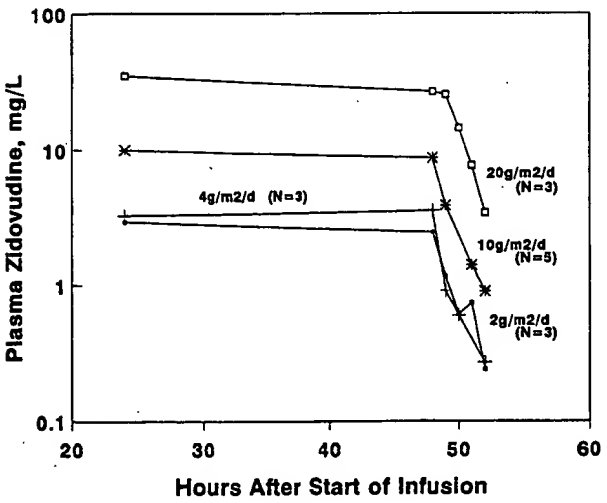

Figure 1. Mean plasma ZDV concentrations for four representative doses (2, 4, 10, and 20 g/m²/day) during 48-hour continuous infusion and during washout.

Table 2. ZDV Pharmacokinetic Parameters for 31 Courses of Therapy

Variable	Mean (± SD)
Total clearance (L/hr)	91.48 ± 56.55
Total clearance (L/hr/kg)	1.44 ± 1.09
V_{area} (L)	169.0 ± 151.3
V_{area} (L/kg)	2.72 ± 2.97
λ_z (1/hr)	0.63 ± 0.21
Half-life (hrs)	1.2 ± 0.6

Results

Pharmacokinetic Analysis

Fourteen patients received 31 courses of therapy. They tended to be elderly, with the primary tumor involving the lower gastrointestinal tract (Table 1). The median serum creatinine was 1.4 mg/dl, with a 3-fold range in individual values.

The ZDV infusions were generally well tolerated. The principal toxicity was mild to severe hyponatremia that was not dose related. It appeared to be secondary to the large amount of free water administered during the ZDV infusion; this effect was observed less frequently when the drug was infused with saline-containing solutions.

Figure 1 depicts the mean plasma ZDV concentrations for four representative doses. The mean

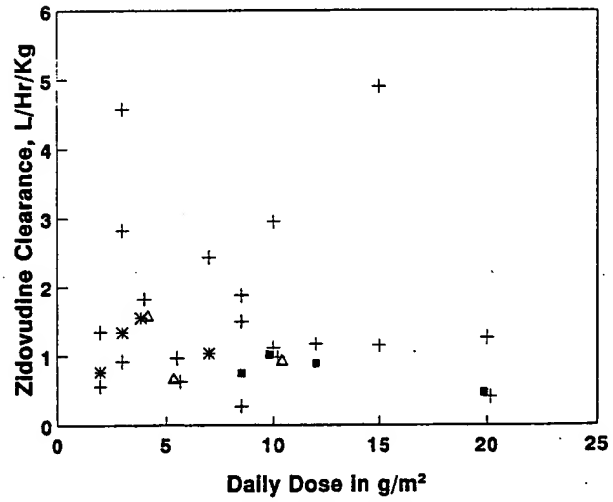

Figure 2. Clearance of ZDV during 31 courses of therapy in 14 patients. Nine patients received more than one course. Values for 11 patients are depicted by (+); individual values for three patients who received several courses with different doses are shown by different symbols (*, Δ, and ■ for each patient) to demonstrate the low degree of intrapatient variability in clearance.

Table 3. Urinary Excretion of ZDV and ZDV Glucuronide (GZDV) and Nonrenal Clearance of ZDV in 5 Patients Receiving 8 Courses of Therapy

Patient Initials	ZDV Dose/ 24 Hours (g/m^2)	Urinary Recovery[a] (%)		GZDV:ZDV[b] Urinary Recovery Ratio	ZDV Renal Clearance (L/hr) During		Nonrenal Clearance (L/hr)[b]
		ZDV	GZDV		Infusion[b]	Washout	
GE	8.5	ND	90	67.5	3.7	6.6	69.3
	15	18	70	6.7	13.1	53.1	75.8
	20	10	90	15.9	4.6	1.8	33.5
JD	10	14	75	8.6	8.2	15.8	53.4
DO	10	11	76	11.9	7.7	7.8	53.1
JM	15	10	75	14.1	11.5	ND	88.4
	20	15	70	6.3	19.3	14.5	77.4
LT	20	8	80	18.2	3.2	8.8	38.1

ND = not done.
[a]Percentage of infused dose recovered as drug in urine between 0 and 52 hours.
[b]From urine collections during the 48-hour infusion.

(± SD) 48-hour (steady-state) concentrations were 2.47 (± 0.5) in three patients receiving 2 g/m^2/day; 3.54 (± 2.4) mg/L in three patients receiving 4 g/m^2/day; 8.72 (± 4.5) mg/L in five patients receiving 10 g/m^2/day; and 26.85 (± 18.6) mg/L in three patients receiving 20 g/m^2/day; the steady-state plasma ZDV levels in patients receiving the highest dose were disproportionally higher than in those receiving lower doses. The postinfusion decline in plasma concentrations was usually best described using a one-compartment pharmacokinetic model.

Considerable interpatient variability was seen in ZDV total clearance (Table 2, Figure 2). In contrast, intrapatient variability appeared to be low, since values for patients receiving several courses at different doses were similar. The relation between AUC and ZDV dose was linear and statistically significant ($p<0.05$; data not shown). Figure 3 shows dose-normalized values of ZDV AUC versus the administered dose; the slope of this relation was not different from zero. However, there was some suggestion of lower clearance with the highest dose (20 g/m^2/day). Clearance values for two courses at the highest dose were among the three lowest observed in this series. Furthermore, one of these patients had received three previous courses of ZDV at lower doses (8.5, 10, and 12 g/m^2/day) and had measured ZDV clearances of 63.7, 86.0, and 73 L/hour, respectively; clearance at 20 g/m^2/day in

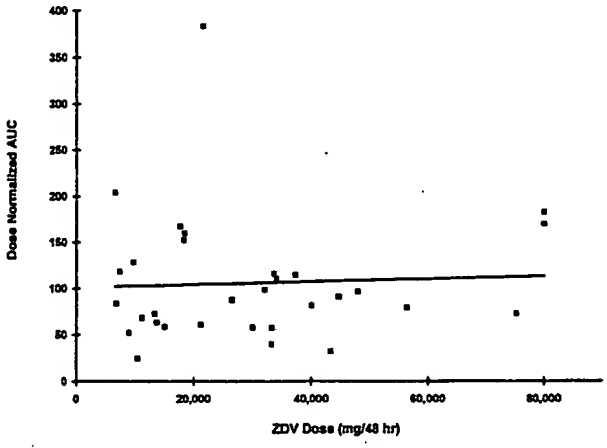

Figure 3. Dose-normalized (to 7000 mg) ZDV AUC according to total dose (in mg) administered over 48 hours. The slope of the line is not statistically significantly different from zero.

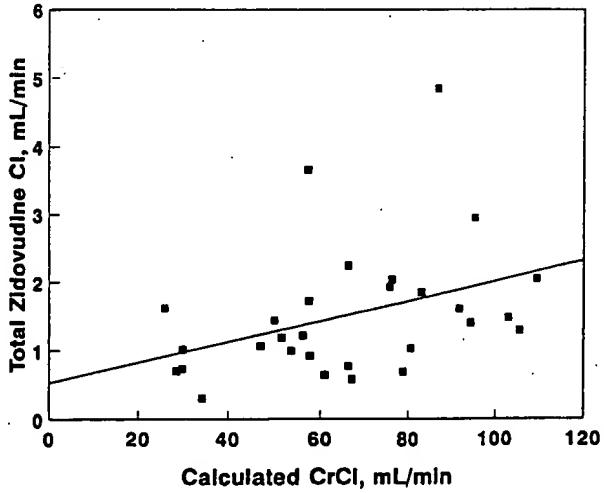

Figure 4. Relation between ZDV clearance and calculated creatinine clearance in 29 courses of therapy in 14 patients. Clearance (in ml/min) = 14.9 • calculated creatinine clearance in ml/min + 526.9; $r^2 = 0.138$, $p<0.05$.

this patient was 38.3 L/hour, or 44–60% of that during previous treatment courses.

The variability in total ZDV clearance was only partly explained by renal function. Figure 4 demonstrates a weak but statistically significant linear correlation between total clearance and calculated creatinine clearance. Table 3 shows the renal excretion of ZDV and ZDV glucuronide in five patients receiving eight courses at doses between 8.5 and 20 g/m²/day. There were no statistically significant differences between renal clearance values measured during or after the infusion. The geometric mean urinary excretion ratio of ZDV glucuronide:ZDV during the 48-hour infusion was 13.6 (range 6.3–67.5); this ratio was similar across all dose levels.

Pharmacodynamic Analysis

The relation between DNA strand breaks and drug exposure in patients receiving 3 (2 patients), 4 (1), 15 (1), and 20 g/m²/day (3) is shown in Figure 5. The percentage of cells with abnormal DNA was best related to ZDV exposure (AUC) by the E_{max} pharmacodynamic model. The sigmoid E_{max} model was also tested, but the estimate for sigmoidicity was not different than 1, so the simpler E_{max} form of the equation was selected.

Discussion

We administered ZDV at daily doses that are over 10-fold greater than those described in previous reports in HIV-infected patients.[15–18] This resulted in steady-state plasma concentrations that were 10–20 times higher than peak levels after usual oral doses for treatment of HIV infection. Despite large differences in doses and resultant concentrations, the pharmacokinetic values of ZDV were comparable with those previously reported after intravenous administration in patients with HIV infection.[15] For example, mean ZDV clearance values were between 1.3 and 2.8 L/kg/hour in 41 patients who received single intravenous doses between 30 and 150 mg over 30 minutes.[18]

Although mean ZDV pharmacokinetics in our patients compare with those reported with lower intravenous doses, two of three patients receiving the highest dose (20 g/m²/day) had markedly lower total clearance, and were among the three lowest clearances reported in all patients. Both renal and nonrenal clearances were reduced in these patients. Whether doses of 20 g/m²/day or higher result in nonlinear pharmacokinetics requires further study.

Zidovudine is predominantly excreted in urine as an ether glucuronide.[15] Since the major route of excretion is by metabolism, the differences in total clearance in our patients were explained only partly by renal function, and were consistent with previous values for the fraction of a dose excreted unchanged in urine (~14%). However, kidney function may also contribute to the metabolic clearance (glucuronidation) of ZDV, as recent studies demonstrated significant formation of zidovudine glucuronide in kidney microsomes.[19]

The considerable variability in in vivo ZDV exposure allowed us to investigate the relation between drug exposure and a marker for incorporation of ZDV into DNA. The E_{max} pharmacodynamic model adequately described the relation between the percentage of abnormal DNA in peripheral white blood cells and ZDV AUC. Estimates using this model indicate that an AUC approximately 4 times greater than that observed with the highest dose of ZDV given in this study would be required to exceed 90% abnormal DNA. If nonlinear pharmacokinetic properties are manifest at higher doses, incrementally smaller increases in dose may be sufficient to attain this target. Alternatively, short infusions of lower doses (and AUC) of ZDV but with high peak levels may give improved results, and are currently being studied.

Previous studies of oral ZDV in patients with HIV infection reported significant interpatient variability in drug clearance (and thus in vivo

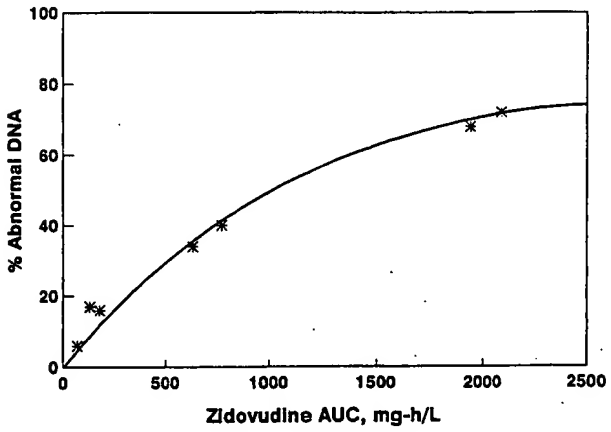

Figure 5. Relation between ZDV AUC and the percentage of abnormal DNA (compared with control) in peripheral white blood cells of seven patients receiving 3–20 g/m²/day. The percentage of cells with abnormal DNA was best described by the E_{max} pharmacodynamic model as % abnormal DNA = AUC •100/(982 + AUC).

exposure as measured by AUC). Our results confirm and extend this observation to patients with cancer receiving large intravenous doses as a continuous infusion. The substantial interpatient variability in clearance but demonstrable relation between ZDV exposure (AUC) and DNA chain-breakage has significant implications for further evaluation of this drug in clinical trials for the treatment of cancer.

Studies of high-dose ZDV may be best conducted controlling drug exposure to achieve target levels of exposure or effects (e.g., % abnormal DNA). This may be important, since pharmacokinetic variability in patients is large and could result in variable antitumor effects and subsequent clinical response. Further studies providing other profiles of drug exposure derived from alternative modes of administration (e.g., bolus dosing) are in progress to elucidate fully the role of ZDV-containing regimens in the treatment of cancer.[20]

References

1. Horowitz J, Chua J, Noel M. Nucleosides. V. The monomesylates of 1-(2ª-deoxy-β-d-oxofuranosyl) thymines. Organ Chem 1964;29:2076–8.
2. Fischl MA, Richman DD, Grieco MH, et al. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. N Engl J Med 1987;317:185–91.
3. Lane HC, Falloon J, Walker RE, et al. ZDV in patients with human immunodeficiency virus (HIV) infection and Kaposi's sarcoma. Ann Intern Med 1983;111:41–50.
4. Brunetti I, Falcone A, Calabresi P, Goulette FA, Darnowski JW. 5-FU enhances azidothymidine cytotoxicity. In vitro, in vivo and biochemical studies. Cancer Res 1990;50:4028–31.
5. Scanlon KF, Kashani-Sabet M, Sowers LC. Overexpression of DNA replication and repair enzymes in cisplatin-resistant human colon carcinoma HCT8 cells and circumvention by azidothymidine. Cancer Communication 1989;1:269–75.
6. Weber G, Ichikawa S, Nagai M, Natsumeda Y. Azidothymidine inhibition of thymidine kinase and synergistic cytotoxicity with methotrexate and 5-fluorouracil in rat hepatoma and human colon cancer cells. Cancer Communication 1990;2:129–33.
7. Paterson ARP, Kolassa N, Cass CE. Transport of nucleoside drugs in animal cells. Pharmacol Ther 1981;12:516–36.
8. Posner MR, Darnowski J, Weitberg A, et al. High-dose intravenous ZDV with 5-fluorouracil, and leucovorin, a phase I trial. Cancer 1992;70:2929–34.
9. Posner MR, Darnowski JW, Calabresi P, et al. Oral azidothymidine, continuous infusion 5-fluorouracil, and oral leucovorin: a phase I study. J Natl Cancer Inst 1990,82: 1710–14.
10. Holford N. MK model, version 4. Cambridge, England: Biosoft, 1990.
11. Schwarz G. Estimating the dimension of a model. Ann Stat 1978;6:461–4.
12. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. Nephron 1976;16:31–41.
13. Snedecor GW, Cochran WG. Statistical methods, 6th ed. Ames: Iowa State University Press, 1967:157–8.
14. LaLonde R. Pharmacodynamics. In: Evans WE, Schentag JJ, Jusko WJ, eds. Applied pharmacokinetics, 3rd ed. Vancouver, WA: Applied Therapeutics, 1992.
15. Dudley MN. Clinical pharmacokinetics of nucleoside antiretroviral agents. J Infect Dis 1995;171(suppl 2):S99–112.
16. Blum MR, Liao ST, Good SS, de Miranda P. Pharmacokinetics and bioavailability of zidovudine in humans. Am J Med 1988;85:189–94.
17. Klecker RW, Collins JM, Yarchoan R, et al. Plasma and cerebrospinal fluid pharmacokinetics of 3′-azido-3′-deoxythymidine, a novel pyrimidine analog with potential application for the treatment of patients with AIDS and related diseases. Clin Pharmacol Ther 1987;41:407–12.
18. Tartaglione TA, Collier AC, Opheim K, Gianola FG, Benedetti J, Corey L. Pharmacokinetic evaluations of low- and high-dose zidovudine plus high-dose acyclovir in patients with symptomatic human immunodeficiency virus infection. Antimicrob Agents Chemother 1991;35:2225–31.
19. Howe JL, Back DJ, Colbert J. Extrahepatic metabolism of zidovudine. Br J Clin Pharmacol 1992;33:190–2.
20. Beitz JG, Darnowski JW, Cummings FJ, et al. A phase I and pharmacologic analysis of fluorouracil and leucovorin combined with infused high-dose zidovudine. Proc Am Assoc Cancer Res 1992;33:427.

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Phase I Trial of High-Dose Infused Zidovudine Combined with Leucovorin plus Fluorouracil

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ABSTRACT

This phase I trial evaluated a high-dose, short-term infusion of zidovudine (AZT) following oral leucovorin (LV) and bolus 5-fluorouracil (FUra). Thirteen patients with metastatic cancer received 30 cycles of therapy. Plasma monitoring demonstrated a dose-dependent increase in peak plasma levels of AZT through the range of dose levels, from 104.3 ± 8.7 μM at the 1.5 g/m² dose of AZT to 1312.6 ± 165.9 μM at the 11.0 g/m² dose. While AZT did not potentiate the usual clinical toxicities of LV plus FUra, an unexpected finding of symptomatic hypotension during the AZT infusion was the dose-limiting toxicity in this trial. One partial response was observed in a previously untreated patient with metastatic colorectal cancer. The maximal tolerated dose of AZT, 7.0 g/m² over 2 hr, is recommended for future phase II evaluation of this novel combination.

INTRODUCTION

Zidovudine (AZT) has synergistic antineoplastic effects when used in combination with 5-fluorouracil (FUra) in human tumor cell lines and tumor xenografts in athymic nude mice (1–3). Importantly, the in vivo synergism has been obtained without increasing toxicity over that of FUra alone. biochemical studies have re-

vealed that this enhanced antineoplastic effect is accomplished through increased incorporation of AZT into DNA in the presence of FUra (3). AZT does not appear to affect the metabolism or activity of FUra (1).

A phase I and clinical pharmacological study evaluating oral AZT given over 2 days with a fixed dose of FUra (800 mg/m^2/day by continuous infusion for 3 days) and oral leucovorin has been performed (4). Eighteen patients with advanced malignancies were treated with doses of AZT ranging from 1.0 to 9.0 g/m^2/day. Nausea and vomiting were dose-limiting, with a maximal tolerated dose of 7.5 g/m^2/day. Although a dose-related increase in plasma levels of AZT was observed, no clinical responses were documented, suggesting that adequate plasma AZT levels might not have been achieved.

A subsequent phase I clinical trial was undertaken using intravenous AZT to deliver a higher dose of drug than possible by the oral route, in an effort to achieve higher plasma levels. Eighteen patients with advanced cancer were treated with the same fixed doses of FUra (800 mg/m^2/day by continuous infusion for 3 days) and oral leucovorin. AZT was infused starting 24 hr after the start of FUra and leucovorin and continued for 48 hr terminating at the end of the FUra infusion. AZT, administered in doses of 2–20 g/m^2/day, added no obvious toxicity to the basic chemotherapeutic treatment with leucovorin and FUra. At doses above 15 g/m^2/day, plasma AZT levels increased dramatically in relation to the dose of AZT. Despite this, no clinical responses were seen in this heavily pretreated group of patients. Limitations in drug delivery restricted administration of higher intravenous doses (5). This report describes a third phase I clinical trial giving AZT as a more rapid (2 hr) infusion, in an attempt to achieve higher peak plasma levels of AZT.

METHODS

Selection of Patients

Patients with a histologically documented diagnosis of carcinoma with clinical and/or laboratory evidence of recurrence and/or disseminated disease were eligible for treatment on this trial. Patients with measurable, bidimensional disease were included. Prior chemotherapy or hormonal therapy, either in the adjuvant setting or for treatment of metastatic disease, was acceptable, assuming the effects of the last therapy had resolved. Patients who had prior radiotherapy were eligible provided radiotherapy did not include >3000 rads to >50% of the pelvic bone structure and lower spine, and the only site(s) of measurable disease were outside the radiotherapy port or had progressed since completion of radiotherapy.

Patients were required to have adequate hematological, renal (serum creatinine $\leq$ 2.0 mg%), and hepatic function (total bilirubin $\leq$ 1.5 mg% and aspartate aminotransferase $\leq$ 2.5 times the upper limit of normal) and an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2. Written consent was obtained prior to therapy.

Treatment Plan

Each cycle consisted of four fixed weekly doses of loading oral leucovorin and intravenous bolus FUra given before AZT was administered as a 2-hr infusion. Patients received four doses of oral LV (100 mg, Wellcovorin^R brand, Burroughs Wellcome Co., Research Triangle Park, NC) hourly up to the anticipated administration of FUra, and two additional doses at 4 and 8 hr after the FUra dose for a total of 600 mg. The FUra dose was 400 mg/m^2 given as an intravenous bolus. One hour after the FUra, the AZT infusion was administered over 2 hr. The AZT was dissolved in sterile water at a concentration of 20 mg/ml (Retrovir^R brand, Burroughs Wellcome Co.) and given directly to patients receiving 7.0 g/m^2 of AZT or less. At higher doses, 0.9% normal saline was added to the AZT to a total volume of 1200 ml prior to infusion. The FUra and AZT were given in an outpatient treatment center, while the LV doses were taken at home. The starting dose of AZT, 1.5 g/m^2 over 2 hr, represented a similar dose rate to the highest dose level seen with a 48-hr continuous infusion of AZT (20 g/m^2/day $\times$ 2 days) (5). The dose of AZT was escalated in 1–2 g/m^2 increments as follows: 3.0, 5.0, 7.0, 9.0, and 11.0 g/m^2. At least 3 patients were treated at each AZT level. Cycles were repeated starting at each fifth week, and patients completing a cycle without toxicity could be escalated to the next AZT dose level.

Study Parameters

Prior to entry onto the protocol, the following information was collected on each patient: a history, physical examination, complete blood count (including differential and platelets), electrolytes, liver function tests (including total bilirubin, alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase), carcinoembryonic antigen (CEA) level (if indicated), chest

X-ray, and the specific radiological studies necessary to define the extent of tumor spread. A complete blood count was performed weekly. All studies performed before entry were repeated at the beginning of each course, except the radiological studies, which were optional.

Response Criteria

After two complete courses of treatment, the patient was evaluated for response according to ECOG criteria. A complete response was defined as complete disappearance of all clinically detectable malignant disease without development of new malignant lesions, lasting for a minimum of 4 weeks. A partial response was defined as greater than or equal to a 50% decrease in tumor size (determined by multiplication of the longest diameter by the greatest perpendicular diameter) for a minimum of 4 weeks. If decreases in bidimensional, measurable disease of less than 50% had occurred, without the appearance of new malignant lesions, for a minimum of 4 weeks, the patient was considered to have stable disease. Finally, patients with a significant increase in the size of lesions present at the start of therapy, or with the appearance of new metastatic lesions known not to be present at the start of therapy, were considered to have had objective progression of their disease.

Termination of Study

Patients were taken off study if objective tumor progression occurred after two courses of treatment or if they developed intolerable or life-threatening toxicity. The study was terminated when a maximum tolerated dose was defined as one dose level below the level that caused a grade IV toxicity in a single patient.

Blood Sampling

Blood samples to monitor plasma AZT were drawn into heparinized tubes from an indwelling intravenous heparin lock in the arm contralateral to the infusion site. Seven-milliliter samples were drawn at four time points each week: 15 min after the FUra was given, immediately before the AZT infusion, at the end of the AZT infusion, and 15 min after the end of the AZT infusion. AZT levels were determined by high-pressure liquid chromatography analysis of plasma separated from cells and processed as previously described (6).

RESULTS

Clinical Findings

Thirteen patients with advanced malignancy completed a total of thirty cycles of treatment on this trial (Table 1). While the majority of patients (85%) had undergone previous treatment for metastatic disease, most maintained an excellent performance status. Seven of the patients had colorectal cancer; 1 of whom had not received prior chemotherapy for metastatic disease. AZT therapy was initiated at 1.5 g/m^2 and escalated in 1.0–2.0 g/m^2 increments to a maximum of 11.0 g/m^2. Three patients were treated at the 1.5 and 3.0 g/m^2 dose levels, 4 at 5.0 g/m^2, and 7 at the 7.0 and 9.0 g/m^2 levels. Only 2 patients were permitted to complete a full cycle of treatment at the 11.0 g/m^2 dose of AZT due to the development of an unexpected grade 4 toxicity in a patient receiving the 9.0 g/m^2 dose concurrently (see below).

Toxicity

No hematological toxicity of ECOG grade 3 or 4 was observed in this trial. Gastrointestinal side effects were manifested as abdominal cramping and diarrhea (Table 2). The severity of these effects was primarily grade 2 or less. In four of 30 (13%) treatment cycles, grade 3

Table 1

Patient Summary

No. of patient entered	13
Sex	
Male	5
Female	8
Age (years)	
Mean	64
Range	40–79
Prior chemotherapy	11
Performance status	
ECOG 0	10
ECOG 1–23	3
Tumor type	
Colorectal	7
Breast	3
Lung	1
Unknown primary	1
Leiomyosarcoma	1
Total cycles	30
Range (per patient)	1–5

Table 2

Incidence of ECOG Grade 3 and 4 Clinical Toxicities

AZT dose (gm/m^2)	GI toxicity # cycles/total cycles	Hypotension # cycles/total cycles
1.5	1[a]/3	0/3
3.0	2/3	0/3
5.0	0/4	1/4
7.0	0/7	6/7
9.0	1/11	7[b]/11
11.0	1/2	2/2
	5/30	16/30

All toxicities reported are ECOG grade 3 except for: [a]one patient with grade 4 abdominal cramping and diarrhea, and [b]one patient with grade 4 hypotension complicated by apnea.

toxicity was observed. In addition, 1 patient experienced grade 4 gastrointestinal toxicity and required hospitalization for intravenous fluids. This patient had been heavily pretreated with many chemotherapeutic agents, including FUra.

By far the most troublesome clinical toxicity was the development of symptomatic hypotension, ECOG grade 3, during the AZT infusion, in 10 of 13 (77%) patients receiving AZT doses of 7.0 g/m^2 or higher. Declines in mean arterial pressure ranged from 10 to 45 mmHg. The hypotension was associated with one or more of the following signs and symptoms: lightheadedness, drowsiness, diaphoresis, nausea, vomiting, headache, back pain, precordial chest pain, and, in one instance, apnea. The frequency of these reactions at each AZT dose level is indicated in Table 2. Precordial chest pain occurred in 2 elderly male patients with a prior history of stable coronary artery disease, 1 treated at 5.0 and the other at 9.0 g/m^2 of AZT. The latter was hospitalized for further cardiological evaluation and suffered no further sequelae, but both patients were taken off study because of this. A single grade 4 toxicity was noted in a 61-year-old woman who suddenly developed apnea and a nonpalpable blood pressure shortly after the start of a 9.0 g/m^2 infusion of AZT. She was resuscitated quickly with chest compressions and ambu bagging and was observed in the hospital for 24 hr before being discharged. This patient had previously received AZT at 7.0 g/m^2 without complications.

Response Evaluation

A partial response was observed in a previously untreated, 77-year-old male patient with colorectal cancer and metastatic disease to the liver. Following one cycle of treatment with 9.0 g/m^2 of AZT, there was a marked decline in the serum CEA level (from 122 to 20) and an 85% decrease in the size of several liver lesions on computed-tomography scan. This patient was taken off study, however, on the first day of the second cycle, due to the development of precordial chest pain during the AZT infusion, necessitating hospitalization. Stable disease was observed in 4 patients in liver, lung, and soft tissue disease sites, who received one to four cycles of therapy. Two of these patients had to be removed from study because of symptomatic hypotension. The remaining 8 patients developed progressive disease.

Plasma Concentration of AZT Following High-dose Infusion

Plasma monitoring demonstrated a predictable, dose-dependent increase in the peak plasma levels of AZT from 104.3 ± 8.7 μM at the 1.5 g/m^2 dose to 1312.6 ± 165.9 μM at the 11.0 g/m^2 dose of AZT (Fig. 1). By this route and schedule, the peak plasma level for the 9.0 g/m^2 dose was 30-fold greater than that reported for the oral 9.0 g/m^2 AZT dose in a previous trial (4).

DISCUSSION

This report presents the clinical and pharmacological results of a phase I trial evaluating the novel combination of escalating, high-dose infusion of AZT with fixed doses of oral leucovorin and bolus FUra in patients with

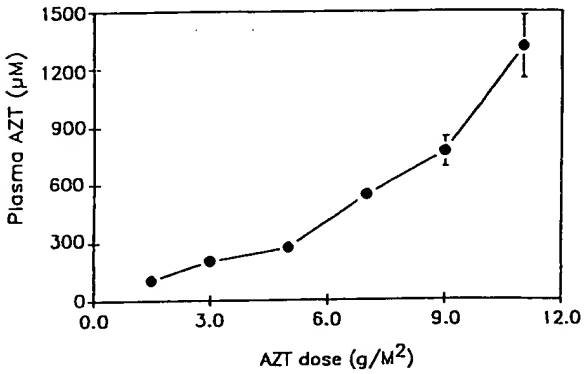

Figure 1. Peak plasma AZT levels as a function of dose. Samples were drawn at the end of the 2-hr AZT infusion, and AZT content was determined by high-pressure liquid chromatography methods. Each point represents the mean and SE of 6–26 determinations.

advanced cancer. Whereas AZT has little intrinsic antineoplastic activity (7), in the presence of agents that inhibit de novo thymidylate production, we postulate that AZT competes for phosphorylation by thymidine kinase and would be increasingly incorporated into DNA, causing premature chain termination (8,9). Moreover, differences between tumor cells and normal tissues in thymidine metabolism exist, such that tumor cells can salvage thymidine more efficiently (10). Thus, the combination of high plasma levels of AZT, in the presence of the thymidylate synthase inhibitor FUra, would be expected to have significant antitumor activity (1–3).

In a previous trial, oral AZT in combination with fixed-dose oral leucovorin (LV) and FUra could be given safely at 7.5 g/m^2/day. However, nausea and vomiting were dose-limiting, and clinically relevant, sustained plasma AZT levels were not achieved (4). Rapid infusion of AZT over 2 hr in the current combination can achieve peak plasma levels found to be effective in preclinical studies. While AZT did not appear to potentiate the expected hematological or gastrointestinal clinical toxicities of LV plus FUra, the unexpected finding of symptomatic hypotension was the dose-limiting toxicity in this trial.

Several approaches were taken to combat the drops in blood pressure that occurred during the AZT infusions. Initially, normal saline boluses given while AZT was still running were attempted, but it became necessary to halt the AZT until systolic blood pressure had risen to at least 90 mmHg. Recovery of blood pressure usually occurred within 1 hr of stopping the AZT. In some cases, the AZT infusion was resumed at a slower rate without further difficulty. At the 7.0 g/m^2 dose level, all patients began to receive premedication with decadron 10 mg/m^2 intravenously, 15 min prior to the AZT, to offset the possible "allergic" component to the reaction. No consistent improvement was noted, however. At dose levels of 9.0 and higher, the AZT was mixed with additional normal saline to a total volume of 1200 ml and administered over 2 hr at a rate of 600 cc/hr. While mean changes in systolic blood pressure were minimized for the 9.0 g/m^2 doses, this measure failed to control blood pressure drops for the patients receiving the 11.0 g/m^2 dose. Symptomatic treatment of any associated symptoms, such as vomiting or pain, was given as indicated.

Although the blood pressure declines were partially ameliorated by the addition of normal saline to the AZT prior to infusion, the maximally tolerated dose in this trial was defined at 7.0 g/m^2 of AZT, that is, one dose level below which a grade 4 hypotensive episode occurred. Therefore, it is recommended that future phase II studies utilize a dose of 7.0 g/m^2 of AZT infused over 2 hr, with the following critical modification: the AZT is to be dissolved directly in normal saline instead of sterile water. Thus, the entire infusion of 1200 ml will be formulated in 0.9 normal saline, rather than 0.25 normal saline (approximately) used in this trial. In addition, patients with preexisting cardiac disease, including myocardial infarction, congestive heart failure, and arrhythmias, would be excluded. To date, 14 patients with metastatic colorectal cancer have been enrolled in a phase II trial conducted at three different sites and have received a dose of 7.0 g/m^2 of AZT formulated as described over a 2-hr period in the outpatient setting. To date, the new AZT formulation has been well tolerated, without the development of symptomatic hypotension (unpublished results).

The significance of the one partial response observed in a previously untreated patient with metastatic colorectal cancer is unclear in this small group of patients, most of whom had progressed on other chemotherapy combinations. Phase II evaluation of this novel combination in a larger number of previously untreated patients with metastatic colorectal cancer will better address the antitumor efficacy of this drug combination. If activity is demonstrated in that trial, future phase III comparisons to other active chemotherapy regimens for colon cancer would be warranted.

ACKNOWLEDGMENTS

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REFERENCES

1. Brunetti I, Falcone A, Calabresi P, et al: 5-Fluorouracil enhances azidothymidine cytotoxicity: In vitro, in vivo and biochemical studies. Cancer Res 50:4026–4031, 1990.
2. Brunetti I, Falcone A, Calabresi P, et al: Fluorouracil enhances azidothymidine antineoplastic activity and incorporation into tissue nucleic acids. Proc Am Assoc Cancer Res 31:2377, 1990.
3. Darnowski JW, Brunetti I, Posner MR, et al: Fluorouracil plus azidothymidine cytotoxicity in vitro: Relationship to cellular thymidine kinase activity. Proc Am Assoc Cancer Res 31:2361, 1990.

4. Posner MR, Darnowski JW, Calabresi P, et al: Oral zidovudine, continuous infusion fluorouracil, and oral leucovorin calcium: A phase I study. J Natl Cancer Inst 82:1710–1714, 1990.
5. Posner MR, Darnowski JW, Weitberg AB, et al: High dose intravenous zidovudine with 5-fluorouracil and leucovorin. Cancer 70:2929–2934, 1992.
6. Calabresi P, Falcone A, St. Clair MH, et al: Benzylacyclouridine reverses azidothymidine-induced marrow-suppression without impairment of anti–human immunodeficiency virus activity. Blood 76:2210–2215, 1990.
7. Yarchoan R, Mitsuya H, Myers CE, et al: Clinical pharmacology of 3'-azido-2'3'-dideoxythymidine (zidovudine) and related dideoxynucleosides. N Engl J Med 321:726–738, 1989.
8. Posner MR, Belliveau JF, Weitberg AB, et al: Continuous infusion cisplatin and bolus 5-fluorouracil in colorectal carcinoma. Cancer Treat Rep 71:975–977, 1987.
9. Scanlon KJ, Kashani-Sabet M, Sowers LC: Overexpression of DNA replication and repair enzymes in cisplatin-resistant human colon carcinoma HCT8 cells and circumvention by azidothymidine. Cancer Commun 1:269–275, 1989.
10. Weber G, Jayaram HN, Pillwein K, et al: Salvage pathways as targets of chemotherapy. Adv Enzyme Regul 26:335–352, 1987.

High-Dose Intravenous Zidovudine with 5-Fluorouracil and Leucovorin

A Phase I Trial

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Background. **The inhibition of pyrimidine metabolism by 5-fluorouracil (5-FU) enhances the anti-cancer effects of zidovudine (formerly called AZT) in in vitro and in vivo model systems without additive toxicity. Zidovudine-induced DNA damage correlates with cytotoxicity.**

Methods. **A Phase I trial of high-dose continuous-infusion intravenous zidovudine therapy in combination with 5-FU and leucovorin therapy was performed. Eighteen patients with advanced malignant tumors were treated with 43 courses of oral leucovorin (50 mg every 4 hours); continuous-infusion 5-FU (800 mg/M²/day) for 72 hours (3 days); and zidovudine, begun 24 hours after the start of 5-FU and leucovorin, for 48 hours, and terminating with the end of the 5-FU infusion. Zidovudine plasma levels and zidovudine-induced DNA damage were assessed.**

Results. **Zidovudine administered in doses of 2–20 g/M²/day, added no obvious toxicity to the basic chemotherapeutic treatment with 5-FU and leucovorin but resulted in a dose-dependent biologic effect manifested by an increase in DNA strand breaks in peripheral blood cells. At doses greater than 15 g/M²/day, altered plasma kinetics of zidovudine were observed; plasma zidovudine levels increased dramatically in relation to the dose of zidovudine. Limitations in drug administration restricted administration of higher intravenous doses without achieving a maximally tolerated dose. No responses were seen in this heavily pretreated population.**

Conclusions. **Based on the results of preclinical studies, plasma zidovudine levels greater than those achieved at the maximal dose (133 μm) are required for increased anti-cancer activity with 5-FU. Additional studies using a bolus or rapid infusion as a method of achieving higher peak levels are indicated. *Cancer* 1992; 70:2929–34.**

Key words: zidovudine, cancer chemotherapy, 5-fluorouracil, pharmacokinetics.

Although highly effective as an antiretroviral agent, zidovudine (formerly called AZT) has little intrinsic antineoplastic efficacy as a single agent.[1–4] Recently, it has been shown that inhibition of pyrimidine metabolism can markedly enhance zidovudine anti-cancer effects in cultured human colon tumor cell lines and in vivo in xenografted human tumor models.[1] Zidovudine antitumor synergism with 5-fluorouracil (5-FU) in vivo in murine models was without additive toxicity.[1] Synergism occurs when de novo thymidylate (dTMP) synthesis is inhibited, for example by 5-FU. Thymidine salvage, which is mediated by thymidine kinase (TK), becomes the preponderant source of dTMP for support of DNA synthesis. TK also phosphorylates zidovudine, which as the triphosphate, AZTTP, competes with dTTP in DNA synthesis.[5] The relatively increased metabolism of zidovudine in the presence of thymidylate synthase (TS) inhibition also is enhanced in tumor cells by their reported lack, or low level, of expression of an active sodium-dependent transport system for thymidine into

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neoplastic, compared with normal, cells.[6,7] As a result, zidovudine is efficiently incorporated into DNA, resulting in DNA strand breaks.[1,8,9] In vitro, zidovudine incorporation and associated DNA damage correlate with cytotoxicity.[9]

Because of these experimental observations, we have initiated clinical trials of zidovudine as a potential anti-cancer drug. In a previous Phase I study, high-dose *oral* zidovudine was studied in combination with 5-FU and leucovorin for toxicity, biologic effects, and pharmacokinetics. Nausea and vomiting were dose limiting but were thought to occur as a result of local gastrointestinal effects of the drug. The combination treatment induced DNA strand breaks in peripheral blood cells, which appeared after a threshold dose. DNA damage did not increase with increasing dose after the threshold level. Analysis of plasma zidovudine pharmacokinetics showed no significant differences with high dose oral therapy in dosages as great as 8.5 g/M^2/day compared with that observed with standard doses used in antiretroviral treatment.[8] Peak plasma levels of zidovudine were significantly lower than those used in murine models to produce enhanced therapeutic effects.[1] These data suggested that larger zidovudine doses and plasma levels are required in humans for antineoplastic effects.

In the current study, we evaluated continuous infusion of high-dose intravenous zidovudine in combination with 5-FU and leucovorin in a Phase I clinical trial. Intravenous zidovudine was used to administer a higher dose of drug than possible by the oral route and achieve high plasma levels. High-dose continuous intravenous zidovudine was well tolerated; there was a dose-dependent increase in DNA strand breaks observed in peripheral blood cells. At zidovudine dosages exceeding 15 g/M^2/day, a nonlinear increase in plasma zidovudine levels was observed; despite this change in kinetics, it was determined that continuous infusion was insufficient to achieve the plasma levels needed for efficacy, and the trial was terminated without achieving dose-limiting toxicity.

Materials and Methods

Patient Selection and Examination

Patients with histologically documented malignant disease and no standard alternative therapy were selected for the study. Patients entered the study at least 4 weeks after previous therapy and after resolution of effects from such therapy. Patients were required to have clinically or radiographically evaluable disease; reasonable renal (creatinine, ≤ 1.6 mg/dl) and hepatic function (liver enzymes, ≤ 10 times normal); an expected survival of 8 weeks or more; an Eastern Cooperative Oncology Group (ECOG) performance status 3 or less; and a signed consent for the experimental treatment approved by the Institutional Review Board. Within 2 weeks before therapy, patients were required to have an assessment of measurable or evaluable disease and baseline laboratory tests. Intraabdominal disease and liver metastases were measured by computed tomography scans; bone metastases were assessed by radiographs and bone scans and were considered as evaluable disease.

Serial physical examinations, radiographs, and serum markers were used to evaluate responses in all eligible patients after the start of the second cycle of therapy. Standard ECOG criteria were used to evaluate responses, response duration, and toxicity.[10]

Treatment

Patients were admitted to the hospital for treatment and received oral leucovorin, 50 mg at the start of and every 4 hours during continuous-infusion 5-FU therapy. 5-FU was given at a dosage of 800 mg/M^2/day as a continuous infusion for 72 hours (3 days). The daily dosage of the 5-FU was formulated in 1 l of 5% dextrose in water and infused during a period of 24 hours; this regimen of 5-FU has been found to result in a 10–20% incidence of Grade 2 mucositis in patients.[8,10] Twenty-four hours after starting 5-FU, patients began receiving intravenous zidovudine. Zidovudine was administered as a continuous infusion for 48 hours, terminating with the end of the 5-FU infusion. Zidovudine was initiated at 2.0 g/M^2/day and escalated in 1–5 g/M^2/day increments through ten dosage levels to a final dosage level of 20 g/M^2/day. Five patients were entered at each level until a dosage level of 10 g/M^2/day was reached, after which only three patients were entered per level. Initially, zidovudine was given in 5% dextrose in water, at a concentration of no greater than 4 g/l. After several episodes of hyponatremia, dosages of 8.5 g/M^2/day or more of zidovudine were prepared in 0.45% sodium chloride (NaCl) and 5% dextrose at a maximal concentration of 15 g/l.

At least three patients were treated at each dose level. If no dose-limiting toxicities were encountered, a patient could be treated at a higher dose level. Treatment cycles were 4 weeks long.

Plasma Drug Pharmacokinetics and Analysis of DNA Strand Breaks

Blood samples were collected from patients with heparin or ethylenediamine tetraacetic acid (EDTA) for plasma zidovudine levels or evaluation of DNA strand breaks, respectively. Heparinized blood was collected

at 24 hours, 48 hours, and 72 hours after the start of 5-FU and at intervals after the infusions were terminated. Zidovudine levels were determined by high-performance liquid chromatography (HPLC) analysis of plasma separated from cells and processed as previously described.[8]

Termination of DNA elongation because of misincorporation of zidovudine was used as a pharmacodynamic measure of in vivo effects. Single strand breaks in DNA from peripheral blood nucleated cells (PBC) were measured before and at 48 hours after the start of the zidovudine infusion in selected patients. A fluorescent analysis of DNA unwinding, modified for whole blood cellular populations by collection in EDTA, and lysis of erythrocytes without additional separation of cells was used to evaluate samples.[8,11] Selected patients had populations separated into peripheral blood mononuclear cells and polymorphonuclear leukocytes for separate evaluation. Several patients had samples tested 24 hours after the completion of the infusion. A healthy volunteer was used to establish the negative control for the strand break analysis, and the results are expressed as the percent of abnormal DNA in the patient's test sample compared with that of the healthy volunteer. Several patients were tested at low and high zidovudine doses. Results from these patients were identical to newly treated patients evaluated at each dose level.

Results

Clinical Results

Eighteen patients were treated and had treatment results that were evaluable for toxicity and response. As shown in Table 1, patients had a wide spectrum of advanced solid tumors, dominated by colorectal carcinomas. Most (66%) patients had received 5-FU as part of previous therapeutic regimens, and all but one patient (who had malignant melanoma) had received previous cytotoxic chemotherapy. One patient did not receive therapy with 4.0 g/M^2/day because of a malignant tumor associated complication. A total of 43 courses were administered.

At a dosage of 5.5 g/M^2/day (level 5), mild hyponatremia was encountered in two patients. At a dosage of 7.0 g/M^2/day (level 6), severe hyponatremia developed in two patients (plasma sodium level, 114 and 119 meq/l) and mild hyponatremia (plasma sodium level, 128 meq/l) in one (normal, greater than or equal to 135 meq/l). This was thought to be secondary to large amounts of free water administration in patients with impaired free water excretion as a result of ascites, reduced serum protein, or mild cardiac dysfunction.

Table 1. Patient Characteristics

No. of patients	18
Median age (range) (yr)	63 (26–78)
Prior chemotherapy	17
Prior 5-fluorouracil therapy	12
Tumor site	
Colon	10
Lung, head and neck	4
Other	4
Responses	0
Courses delivered	43
Toxicity (≥ Grade 2)	
Mucositis	9
Nausea/vomiting	9
Hyponatremia	11
Leukopenia	1
Fever	9

Thereafter, zidovudine was prepared in 0.45% NaCl/5% dextrose in water at a concentration that was 3.75 times greater (15 g/l) to ameliorate the problem. Subsequently, only occasional, mild hyponatremia was encountered in a nondose-dependent manner, preponderantly in patients with underlying problems of fluid balance, such as patients with congestive heart failure or ascites.

Mucositis was not dose-dependent, and occurred at Grade 2 or 3 in 9 of 43 (20%) cycles. One patient had a leukocyte count of less than 2000/μl while receiving 7.0 g/M^2/day. No other episodes of leukopenia or thrombocytopenia were encountered. Grade 2 nausea and vomiting also occurred during nine courses and were unrelated to zidovudine dose. Skin rash occurred in one patient and was mild. No patients had nephrotoxicity, neurotoxicity, or hepatobiliary abnormalities as a result of therapy. Four patients had nine acute febrile reactions during zidovudine infusions. In two patients, fever began immediately upon the start of zidovudine, and reoccurred with all subsequent treatments. Maximum temperature was 38.8°C. Febrile reactions were controlled with acetaminophen. No patients in this heavily pretreated and generally unresponsive population showed evidence of a clinically significant response.

Pharmacokinetics of High-Dose Intravenous Zidovudine

Steady-state plasma zidovudine concentrations measured at 24 hours after the start of the infusion are shown in Figure 1. Steady-state concentrations were highly variable between patients but generally increased at a linear rate with dosages as great as 15 g/M^2/day. However, two of three patients who received

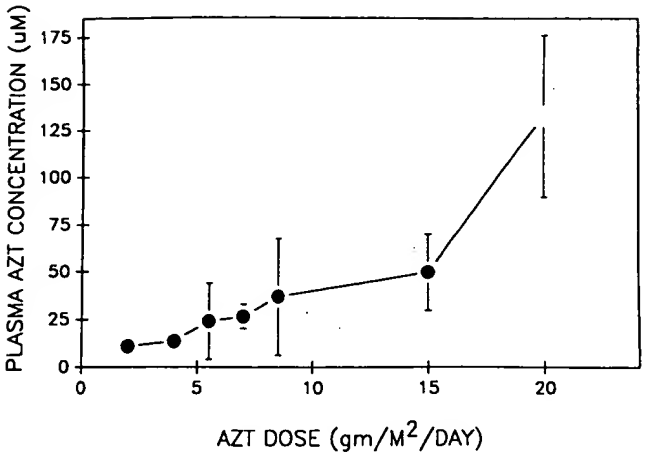

Figure 1. Steady-state plasma zidovudine levels (μM) measured at 24 hours increased with increasing daily dose of continuous-infusion zidovudine.

20 g/M^2/day had markedly higher serum zidovudine concentrations (> 110 μm), suggesting that for those patients zidovudine clearance was reduced compared with other patients who received smaller dosages. The steady-state plasma level achieved at the dosage of 20 g/M^2/day was 133 plus or minus 43 μm (three patients) compared with 50 plus or minus 20 μm at 15 g/M^2/day.

DNA Strand Breaks

The frequency of DNA single strand breaks in peripheral blood cells was evaluated as a measure of the biologic effect of zidovudine. As shown in Figure 2, DNA strand breaks in peripheral blood cell populations occurred in a dose-dependent manner. Before zidovudine therapy, DNA strand breaks of patients were quantitatively identical to those of healthy volunteers, indicating no cumulative effect or pre-existing strand breaks related to prior treatment. In addition, intensity of prior treatment had no apparent effect on zidovudine-induced DNA strand breaks in the small number of patients tested. To determine if DNA damage were more pronounced in different peripheral blood cell populations, PBC from patients treated with 15 g/M^2/day were separated into peripheral blood mononuclear cells and polymorphonuclear cells by density centrifugation and assayed for DNA damage. No differences were observed in the amount of damage detected in either mononuclear cells (preponderantly lymphocytes) or polymorphonuclear cells (data not shown). We also quantitated DNA damage 24 hours after termination of the infusion at 15 g/M^2/day to determine if additional DNA damage or repair might be occurring at this later time point. The amount of DNA damage present in whole peripheral blood cell populations was unchanged 24 hours after completion of the infusion (data not shown).

Discussion

In this study, we report the results of a Phase I trial of high-dose continuous-infusion intravenous zidovudine in combination with 5-FU and leucovorin. High-dose zidovudine added no obvious toxicity to the basic chemotherapeutic treatment with 5-FU and leucovorin but exerted a biologic effect that was manifested by a dose-dependent increase in DNA strand breaks in peripheral blood cells. At the highest doses tested, the clearance of zidovudine appears to change, resulting in a disproportionate increase in plasma zidovudine in relation to the escalation in the dose of zidovudine given.[12] Nonetheless, to increase zidovudine plasma levels to those found to be effective in murine models (500–1000 μm), substantially higher doses of zidovudine would have to be given. As a result of these observations, this Phase I study of continuous-infusion zidovudine therapy was terminated despite a maximally tolerated dose not being achieved.

Previous studies have demonstrated that inhibition of pyrimidine metabolism can enhance zidovudine antineoplastic effects in vitro and in vivo.[1,5,13] Synergistic inhibition of tumor growth was observed in xenografted human colon tumors in nude mice treated with weekly intraperitoneal 5-FU and zidovudine, compared with treatment with 5-FU or zidovudine alone.[1] This effect occurred only when 5-FU preceded zidovudine therapy. Despite impressive antitumor effects, there

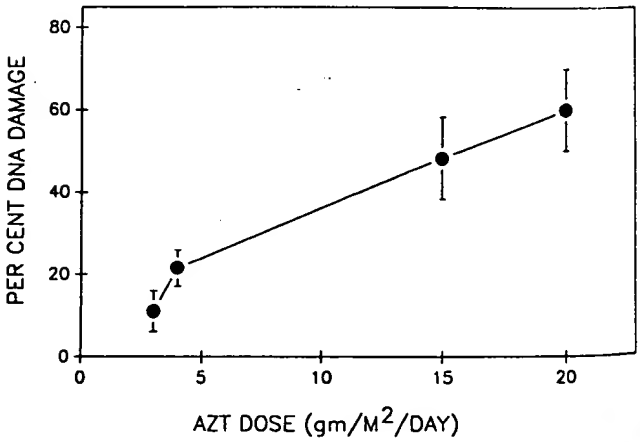

Figure 2. DNA strand breaks, presented as percent of DNA damaged and measured after 48 hours of zidovudine infusion. increased in a dose-dependent manner. The results are expressed as the percent of abnormal DNA in the patient test sample compared with that of the healthy control subject.

was no additive effect on toxicity. The mechanisms that account for synergistic antitumor and tissue-sparing effects are being investigated by several groups.

Weber et al.[3,14] have presented evidence that suggests that zidovudine-related cytotoxicity in human tumor cells is a reflection of the ability of zidovudine to competitively inhibit TK. When de novo dTMP synthesis is inhibited with an agent such as 5-FU, the combined effect of these agents is to significantly inhibit dTMP synthesis, leading to cell death. In contrast, biochemical studies from our group indicate that the degree to which zidovudine is incorporated into DNA is associated with cytotoxicity.[5,9] Presumably in the presence of TS inhibition, intracellular pools of dTTP are reduced, but AZTTP pools are unaffected. Our studies indicate that this imbalance leads to increased incorporation of AZTTP into tumor cell DNA.[9] Indeed, in vivo experiments have demonstrated a preferential incorporation of zidovudine in tumor as opposed to normal murine tissues in the presence of 5-FU.[1,5] Once it is incorporated into DNA, zidovudine induces chromosomal strand breaks and chain termination. The incorporation of zidovudine into DNA directly correlates with in vitro cytotoxicity.[9]

Other manipulations that increase zidovudine metabolism and incorporation into DNA include biochemical modulation of dTMP synthesis at other sites, for example by methotrexate and other inhibitors of dihydrofolate reductase, or increased requirements for dTMP induced by agents such as cisplatin.[2,13] As would be predicted, substantial synergistic antitumor effects have been shown for methotrexate in murine models.[13,14] Scanlon and colleagues[2,15] showed that zidovudine can circumvent cisplatin resistance in an ovarian carcinoma cell line and methotrexate resistance in a human leukemia cell line. Thus, it appears from a variety of studies that zidovudine can provide a means of abrogating resistance to specific agents that alter pyrimidine metabolism. Other more theoretical applications include combination with agents that enhance TK expression or reduce TS activity. These effects occur with interferons and some growth factors.[16] Synergistic antitumor effects of gamma-interferon and 5-FU, for example, have been related to the down regulation of a compensatory increase in TS after 5-FU administration.[16]

Because antitumor effects are difficult to assess in this study design and because preclinical models suggest that no additional toxicity with zidovudine could be expected, DNA strand breaks occurring in PBC in vivo were used to assess biologic activity. PBC strand breaks have been investigated for cisplatin-induced adducts and after treatment with 2-chlorodeoxyadenosine, agents in which response can be correlated with DNA damage.[17,18] The DNA strand break data in this study support the assertion that zidovudine in combination with TS blockade by 5-FU induces a dose-dependent biologic effect in humans. However, these measurements serve only as a surrogate for dose effects in the absence of toxicity.

Plasma zidovudine concentrations in the patients studied suggest that zidovudine dosages exceeding 15 g/M^2/day result in reduced zidovudine clearance. Studies of lower doses of zidovudine show it is metabolized in vivo to an ether glucuronide, with approximately 75% and 60% of an oral or IV dose, respectively, excreted as the conjugate in urine.[19–21] In previous studies with high-dose (1–9 g/M^2/day) oral therapy, increasing oral doses did not result in appreciably higher serum concentrations.[8] This may reflect saturation of facilitated drug transport across intestinal membranes, which has been shown in animals for several other nucleosides and has been noted in humans with zidovudine at lower doses.[22,23] Zidovudine also undergoes significant presystemic (i.e., first-pass) metabolism, thus additionally reducing the amount of drug reaching the peripheral circulation.[17–19] These data and the current observations confirm the high interpatient variability in pharmacokinetic properties of zidovudine. These data also suggest that incrementally smaller dosages of zidovudine greater than 20 g/M^2/day will result in significantly larger changes in steady-state zidovudine concentrations; thus, it is difficult to forecast the dose required to achieve concentrations comparable to those observed in the murine model. In addition, limitations in drug administration limit administration of higher intravenous doses. Plasma zidovudine concentration monitoring and adaptive control of infusion rate may be helpful in achieving target exposure levels.

Because in preclinical murine models bolus intraperitoneal zidovudine achieves plasma levels of 500–1000 μm, substantially greater than the in vivo levels achieved in this study, the failure to observe toxicity or antitumor effects may be explained by the relatively lower plasma zidovudine levels achieved.[1] Higher plasma levels than those achieved by a continuous infusion may be achieved by administration of a rapid infusion. The pharmacokinetic evaluation of 48-hour continuous-infusion zidovudine therapy suggests that this is an impractical method of administration with 5-FU. The theoretical application of zidovudine as a selective and potentially nontoxic means of circumventing resistance to 5-FU and other agents that perturb pyrimidine metabolism lend substantial impetus to the exploration of other modes of administration or combinations. This study highlights the effects of pharmacokinetics and surrogate markers on the performance of clinical trials.

Refer nces

1. Brunetti I, Falcone A, Calabresi P, Goulette FA, Darnowski JW. 5-Fluorouracil enhances azidothymidine cytotoxicity: in vitro, in vivo and biochemical studies. *Cancer Res* 1990; 50:4028–31.
2. Scanlon KF, Kashani-Sabet M, Sowers LC. Overexpression of DNA replication and repair enzymes in cisplatin-resistant human colon carcinoma HCT8 cells and circumvention by azidothymidine. *Cancer Commun* 1989; 1:269–75.
3. Weber G, Ichikawa S, Nagai M, Natsumeda Y. Azidothymidine inhibition of thymidine kinase and synergistic cytotoxicity with methotrexate and 5-fluorouracil in rat hepatoma and human colon cancer cells. *Cancer Commun* 1990; 2:129–33.
4. Lane HC, Falloon J, Walker RE, Deyton L, Kovacs J, Masur H, et al. Zidovudine in patients with human immunodeficiency virus (HIV) infection and Kaposi's Sarcoma. *Ann Intern Med* 1983; 111:41–50.
5. Darnowski JW, Goulette FA. Fluorouracil increases azidothymidine cytotoxicity by altering dTTP pools. *Proc AACR* 1992; 33:2511.
6. Paterson ARP, Kolassa N, Cass CE. Transport of nucleoside drugs in animal cells. *Pharmacol Ther* 1981; 12:516–36.
7. Wohlhueter RM, Marz R, Plagemann PGW. Thymidine transport in cultured mammalian cells: kinetic analysis, temperature dependence and specificity of the transport system. *Biochim Biophys Acta* 1979; 553:262–83.
8. Posner MR, Darnowski JW, Calabresi P, Brunetti I, Corvese D, Curt G, et al. Oral azidothymidine, continuous infusion 5-fluorouracil and oral leucovorin: a phase I study. *J Natl Cancer Inst* 1990; 82:1710–4.
9. Darnowski JW, Renaud CA, Chu M, Goulette F, Tosi P, Calabresi P. Resistance to azidothymidine cytotoxicity in the human colon tumor cell line HCT15 is associated with enhanced removal of AZT from cellular DNA. *Proc AACR* 1991; 32:2124.
10. Posner MR, Martin A, Slapak CA, Clark JW, Cummings FJ, Robert NJ, et al. A phase II trial of continuous infusion cisplatinum and 5-fluorouracil with oral calcium leucovorin in colorectal carcinoma. *Am J Clin Oncol* 1992; 15:239–41.
11. Weitberg AB, Corvese D. Hydroxy- and hydroperoxy-6,8,11,14-eicosatetraenoic acids induce DNA strand breaks in human lymphocytes. *Carcinogen* 1989; 10:1029.
12. Rowley KA, Dudley MN, Darnowski JW, Posner MR. Pharmacokinetics of high dose zidoudine administered as a constant infusion. *Proc ICAAC* 1991; 31:A759.
13. Tosi P, Calabresi P, Goulette FA, Renaud CA, Darnowski JW. Methotrexate enhances 3'-azido-3'deoxythymidine antineoplastic activity. *Proc AACR* 1991; 32:2032.
14. Weber G, Nagai M, Prajda N, Nakamura H, Szekeres T, Olah E. AZT: a biochemical response modifier of methotrexate and 5-fluorouracil cytotoxicity in human ovarian and pancreatic carcinoma cells. *Cancer Commun* 1991; 3:127–32.
15. Newman EM, Miyachi H, Reiling C, Jiao L, Sowers LC, Scanlon KJ. Increased sensitivity to azidothymidine in a subline of CCRF-CEM human leukemia cells resistant to methotrexate. *Proc AACR* 1991; 32:2457.
16. Chu E, Zinn S, Boarman D, Allegra CJ. Interaction of gamma interferon and 5-fluorouracil in the H630 human colon cancer cell line. *Cancer Res* 1990; 50:5834–40.
17. Reed E, Ostchega Y, Steinberg S, Yuspa SH, Young RC, Ozols RF, et al. Evaluation of platinum-DNA adduct levels relative to known prognostic variables in a cohort of ovarian cancer patients. *Cancer Res* 1990; 50:2256–60.
18. Piro LD, Carrera CJ, Beutler E, Carson DA. 2-Chlorodeoxyadenosine: an effective new agent for the treatment of chronic lymphocytic leukemia. *Blood* 1988; 72:1069–73.
19. Yarchoan R, Mitsuya H, Myers CE, Broder S. Clinical pharmacology of 3'-azido-2''3''-dideoxythymidine (zidovudine) and related dideoxynucleosides. *N Engl J Med* 1989; 321:726–38.
20. Balis FM, Pizzo PA, Murphy RF, Eddie J, Jarosinski PF, Falloon J, et al. The pharmacokinetics of zidovudine administered by continuous infusion in children. *Ann Intern Med* 1989; 110:279–85.
21. Collins JM, Unadkat JD. Clinical pharmacokinetics of zidovudine: an overview of current data. *Clin Pharmacokinet* 1989; 17:1–9.
22. Vijayalakshimi J, Belt JA. Sodium-dependent nucleoside transport in mouse intestinal epithelial cells. *J Biol Chem* 1988; 263:19419–23.
23. Blum MR, Liao SHT, Good SS, de Miranda P. Pharmacokinetics and bioavailability of zidovudine in humans. *Am J Med* 1988; 85:189–94.

In conclusion, LED has clinical activity against human breast cancer and appears to be less cardiotoxic than the free drug. These data present numerous avenues for further investigation.

References

(1) GUNVEN P, THEVE NO, PETERSON C: Serum and tissue concentrations of doxorubicin after IV administration of doxorubicin or doxorubicin-DNA complex to patients with gastrointestinal cancer. Cancer Chemother Pharmacol 17:153–156, 1986

(2) MYERS CE, MCGUIRE WP, LISS RH, ET AL: Adriamycin: The role of lipid peroxidation in cardiac toxicity and tumor response. Science 197:165–167, 1977

(3) DOROSHOW JH, LOCKER GY, IFRIM I, ET AL: Prevention of doxorubicin cardiotoxicity in the mouse by *N*-acetylcysteine. J Clin Invest 68:1053–1064, 1981

(4) HERMAN EH, FERRANS VJ, JORDAN W, ET AL: Reduction of chronic daunorubicin cardiotoxicity by ICRF 187 in rabbits. Res Commun Chem Pathol Pharmacol 31:85–97, 1981

(5) SPEYER JL, GREEN MD, KRAMER E, ET AL: Protective effect of bispiperazinedione ICRF-187 against doxorubicin-induced cardiac toxicity in women with advanced breast cancer. N Engl J Med 319:745–752, 1988

(6) RAHMAN A, KESSLER A, MORE N, ET AL: Liposomal protection of adriamycin-induced cardiotoxicity in mice. Cancer Res 40:1532–1537, 1980

(7) RAHMAN A, MORE N, SCHEIN PS: Doxorubicin-induced chronic cardiotoxicity and its protection by liposomal administration. Cancer Res 42:1817–1825, 1982

(8) RAHMAN A, WHITE G, MORE N, ET AL: Pharmacological, toxicological and therapeutic evaluation in mice of doxorubicin entrapped in cardiolipin liposomes. Cancer Res 45:796–803, 1985

(9) RAHMAN A, FUMAGALLI A, BARBIERI B, ET AL: Antitumor and toxicity evaluation of free doxorubicin and doxorubicin entrapped in cardiolipin liposomes. Cancer Chemother Pharmacol 16:22–27, 1986

(10) HERMAN E, RAHMAN A, FERRANS VJ, ET AL: Prevention of chronic doxorubicin cardiotoxicity in beagles by liposomal encapsulation. Cancer Res 43:5427–5432, 1983

(11) RAHMAN A, TREAT J, ROE JK, ET AL: A phase I clinical trial and pharmacokinetic evaluation of liposome-encapsulated doxorubicin. J Clin Oncol 8:1093–1100, 1990

(12) BRISTOW MR, MASON JW, BILLINGHAM ME, ET AL: Doxorubicin cardiomyopathy: Evaluation by phonocardiography, endomyocardial biopsy, and cardiac catheterization. Ann Intern Med 88:168–175, 1978

(13) BILLINGHAM ME, MASON JW, BRISTOW MR, ET AL: Anthracycline cardiomyopathy monitored by morphologic changes. Cancer Treat Rep 62:865–872, 1978

(14) HOOGSTRATEN B: Adriamycin (NSC-123127) in the treatment of advanced breast cancer: Studies of the Southwest Oncology Group. Cancer Chemother Rep Part 3, vol 6:329–334, 1975

(15) JULIANO RL, STAMP D: Pharmacokinetics of liposome-encapsulated antitumor drugs. Biochem Pharmacol 27:21–27, 1988

(16) TORMEY D: Adriamycin (NSC-123127) in breast cancer: An overview of studies. Cancer Chemother Rep Part 3, vol 6:319–327, 1975

(17) DRUCK M, GULEVCHYN K, EVANS W, ET AL: Radionuclide angiography and endomyocardial biopsy in the assessment of doxorubicin cardiotoxicity. Cancer 53:1670–1674, 1984

(18) POTKUL RK, TREAT J, FORST D, ET AL: A phase I-II study of intraperitoneal (IP) administered liposome-encapsulated doxorubicin (LED). Proc ASCO 8:312, 1989

Oral Zidovudine, Continuous-Infusion Fluorouracil, and Oral Leucovorin Calcium: A Phase I Study

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A phase I clinical, pharmacologic, and biochemical evaluation of escalating oral zidovudine (AZT) given over 2 days with a fixed dose of continuous-infusion fluorouracil (800 mg/m² per day × 3 days) and oral leucovorin calcium was performed. Eighteen patients were treated with doses of AZT ranging from 1.0 to 9.0 g/m² per day. Nausea and vomiting were dose limiting, with a maximally tolerated dose of 7.5 g/m² per day. Rash and mucositis occurred but were not dose limiting. A dose-related increase in peak plasma levels of AZT was observed, and the alpha half-life of AZT in plasma (75 min) was unaffected by these high doses. At doses above 4.0 g/m² per day, trough levels significantly increased, perhaps reflecting prolonged absorption from the gut. No responses were observed; however, a significant increase in DNA single-strand breaks was observed in peripheral blood cells after a threshold dose of 4.0 g/m² per day, confirming a biological effect of AZT in this regimen. Further trials with an intravenous formulation capable of maintaining plasma levels and circumventing dose-limiting toxicity are warranted. [J Natl Cancer Inst 82:1710–1714, 1990]

Fluorouracil (5-FU) is the most active single agent in the treatment of colorectal carcinoma and has reproducible activity in other solid tumors as well. The limited effectiveness of 5-FU, however, has encouraged the development of strategies to modulate and enhance the antitumor activity of this and related agents (*1,2*). Recent efforts have focused on biochemically modulating 5-FU inhibition of thymidylate synthase by means of the addition of reduced folates (*3*). Increasing thymidylate synthase inhibition, and thus restricting thymidylate (dTMP) available for DNA synthesis, has led to small increases in clinical response rates and survival in patients with resistant tumors (*4*).

An alternative approach to the biochemical modulation of 5-FU would be to exploit differences between normal and neoplastic cells in their ability to salvage pyrimidine nucleosides. Human tumors have a greater capacity to salvage thymidine by means of thymidine kinase (TK) than do normal tissues (*5,6*). In addition, unlike normal cells, tumor cells appear to be unable to actively transport pyrimidine nucleosides from plasma, leading to relatively low intracellular thymidine levels (*7,8*). The combination of 5-FU and leucovorin calcium administration to inhibit de novo dTMP synthesis with the use of an antitumor agent activated through TK might be expected to have enhanced antineoplastic effects. Biochemical studies in our laboratory indicate that zidovudine (AZT), a thymidine analogue that is salvaged in a manner analogous to that of thymidine and then incorporated into viral DNA by reverse transcriptase during retroviral RNA-mediated DNA formation, is such an agent (*9–11*).

We have recently reported that AZT has synergistic antineoplastic effects when used in combination with 5-FU in several human tumor cell lines and human tumor xenografts in athymic

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nude mice (*9–11*). Importantly, the in vivo synergism has been obtained without increasing toxicity over that of 5-FU alone. Biochemical studies reveal that this enhanced antineoplastic effect is accomplished through increased incorporation of AZT into DNA in the presence of 5-FU (*11*). AZT does not appear to affect the metabolism or activity of 5-FU (*9*).

In this report, we describe a phase I clinical, pharmacologic, and biochemical evaluation of increasing oral AZT (1.0–9.0 g/m^2 per day) in combination with a fixed dose of continuous-infusion 5-FU and oral leucovorin used to biochemically modulate the antineoplastic activity of AZT. Dose-limiting toxicity was nausea and vomiting. Rash and mucositis occurred, and mucositis increased with increasing dose but was not dose limiting. A dose-dependent increase in peak and trough plasma levels of AZT was observed; however, dose-limiting nausea and vomiting at the highest doses administered appeared to affect these levels. A significant increase in DNA strand breaks was observed in peripheral blood cells, possibly after a threshold trough plasma AZT level (10 μM) was achieved, confirming a biological effect of AZT in this regimen.

Patients and Methods

Patient Selection, Treatment, and Evaluation

Criteria for acceptance into treatment were the following: histologically documented malignancy with no standard alternative therapy; passage of at least 4 weeks since, and resolution of effects from, the last therapy; disease clinically or radiographically assessable; reasonable renal (creatinine ≤1.6 mg/dL) and hepatic function (liver enzymes ≤10 times normal); an expected survival of 8 weeks or more; an Eastern Cooperative Oncology Group (ECOG) performance status less than or equal to 3; and a signed consent for the experimental treatment approved by the Institutional Review Board. Within 2 weeks prior to therapy, patients were required to have an assessment of measurable or assessable disease and baseline laboratory tests. Intra-abdominal disease and liver metastases were measured by computed tomographic scans; bone metastases were assessed by radiographs and bone scans and were considered to constitute assessable disease.

Patients were admitted to the hospital for treatment and received oral leukovorin (50 mg) 2 hours before and every 4 hours during continuous infusion of 5-FU, 5-FU was given at a dose of 800 mg/m per day as a continuous infusion for 72 hours (3 days). The daily dose of the 5-FU was formulated in 1 L of 5% dextrose in water and infused over 24 hours. This regimen of 5-FU has been found to result in a 10%–20% incidence of grade 1 mucositis in patients. Twenty-four hours after starting infusion of 5-FU, patients began taking oral AZT, which was provided as commercially available 100-mg capsules (Retrovir; Burroughs Wellcome, Research Triangle Park, NC). One sixth of the daily dose of AZT was given every 4 hours as capsules or dissolved in orange juice. Patients received AZT with 5-FU for 48 hours. 5-FU infusion was completed after a total of 72 hours of continuous infusion; however, patients received an additional dose of AZT and leucovorin 4 hours later. Five patients were planned for each level, and patients completing a cycle could be escalated to the next test level. Additional cycles were planned at the maximally tolerated dose level. Treatment was re-initiated each 28 days unless altered by drug-induced toxicity.

Serial physical examinations, radiographs, and serum markers were used to evaluate responses in all eligible patients after the start of the second cycle of therapy. Standard ECOG criteria were used to evaluate responses, response duration, and toxicity (*12*).

Plasma-Drug Pharmacokinetics and Analysis of DNA Strand Breaks

Blood samples were collected from study patients with heparin or edetic acid (EDTA) for determination of plasma AZT levels or evaluation of DNA strand breaks, respectively. Blood was collected immediately prior to and 24, 48, and 72 hours after the start of 5-FU infusion as well as prior to and every hour after the last dose of oral AZT for 4 hours. AZT levels were determined by high-pressure liquid-chromatography analysis of plasma separated from cells and processed as previously described (*13*). DNA single-strand breaks in peripheral blood-nucleated cells were monitored prior to and at the end of administration of oral AZT using the fluorescent analysis of DNA unwinding modified for whole-blood cellular populations by collection in EDTA and lysis of red blood cells without further separation of cells (*14,15*). A normal volunteer was used to establish the negative control for the strand-break analysis, and the results were expressed as a percentage of normal DNA in the test sample compared with 100% for the normal negative control.

Results

Clinical Results

Eighteen patients were entered into this clinical trial. As shown in table 1, the tumors in these patients represented a wide spectrum of advanced solid tumors dominated by colorectal and lung carcinomas. The majority of patients (72%) had received 5-FU as part of previous therapeutic regimens. AZT therapy was initiated at 1.0 g/m^2 per day and escalated in 0.5- to 1.5-g/m^2-per-day increments as planned. Five patients were entered at each level, with few exceptions. One patient did not receive therapy with 2.0 g/m^2 per day due to a malignancy-associated complication. Nine patients were treated with the maximally tolerated dose, 7.5 g/m^2 per day. Three patients were treated with the next highest dose, 9.0 g/m^2 per day.

Of importance is that no leukopenia or thrombocytopenia was encountered. As can be seen in table 2, toxic effects were limited to mucositis, rash, and nausea and vomiting. Nausea and vomiting of 3–4 days' duration were dose limiting despite aggressive therapy with

Table 1. Patient characteristics*

No. of patients entered	18
Sex	
Male	9
Female	9
Age (yr)	
Median	59
Range	39–79
Prior chemotherapy	18
Prior 5-FU	13
Performance status	
Median	1
Range	0–3
Tumor site or type	
Colon	6
Lung	4
Breast	3
Melanoma	2
Pancreas	1
Esophagus	1
Head and neck	1
Total cycles	
Mean per patient	50
Median per patient	2.8
Range	1–8

*Unless otherwise specified, values = No. of patients.

Table 2. Toxicity of therapy

AZT dose level (g)	Patients*	Toxicity†							
		Mucositis			Rash			Nausea and vomiting	
		1	2	3	1	2	3	1	2
1.0	5	1	1	0	0	1	0	1	0
1.5	5 (2)	1	0	0	0	0	0	0	0
2.0	4 (2)	2	0	1	0	1	0	2	0
3.0	5 (1)	2	0	0	0	0	0	2	1
4.0	5 (1)	3	0	0	0	0	0	0	0
5.0	5 (2)	1	3	0	0	0	2	0	3
6.0	5 (2)	2	2	0	1	0	0	1	0
7.5	9 (3)	6	0	0	0	2	0	2	1
7.5‡	4	1	1	1	2	0	0	1	0
9.0	3	1	2	0	1	1	0	0	0

*Patients entered (new patients).
†ECOG grades 1–3.
‡Represents a second treatment in patients having no toxic effects.

intravenous anti-emetics. Four of nine patients experienced intractable nausea and vomiting at 7.5 g/m² per day of AZT on either the first (three of nine) or a subsequent (one of four) treatment at this dose. Two of three patients who tolerated 7.5 g/m² per day and were subsequently treated at 9.0 g/m² per day developed intractable vomiting at the higher dose. Occasional mucositis occurred at all dose levels. As the maximum tolerable oral dose was approached, mucositis increased in frequency and appeared in patients who had tolerated lower doses of AZT without this toxicity. It is noteworthy that two of three patients treated at 9.0 g/m² per day, without previous mucositis at the next lower dose, developed grade 1 or 2 mucositis. Mucositis began on day 3 or 4 of treatment and resolved by days 11–14. Rashes appeared as desquamative or erythematous skin eruptions and occurred more frequently in previously irradiated skin. There was no dose dependency for the development of rashes. One patient developed extensive peeling of the hands at 7.5 g/m² per day similar to the hand–foot syndrome seen with prolonged 5-FU infusions. No patients developed nephrotoxicity, neurotoxicity, or hepatobiliary abnormalities as a result of therapy. Two patients experienced serious urosepsis associated with nephrostomy tubes previously placed for malignant ureteral obstruction.

No patient treated at the doses employed in this trial showed evidence of a response. In one patient with previously treated colorectal carcinoma, a lung metastasis showed over 50% regression, although hepatic metastases progressed.

Pharmacokinetics of High-Dose Oral AZT

Pharmacokinetic studies demonstrated a predictable, dose-dependent increase in the peak plasma levels of AZT to 26 ± 9 μ*M* at 5.0 g/m² per day. As shown in figure 1, however, above this dose, absorption of AZT appeared to be erratic. Trough levels also increased but appeared to undergo a stepwise change that may have been related to prolonged and delayed absorption from the gut at doses at or above 5.0 g/m² per day. The plasma clearance of AZT at 7.5 g/m² per day appeared to be biphasic, perhaps reflecting this prolonged absorption from the gut and subsequent plasma elimination. The alpha plasma half-life at this dose level, as shown in the inset to figure 1, was 75 minutes and was not significantly different from that reported in the literature for much lower oral doses.

DNA Strand Breaks

The frequency of DNA single-strand breaks in peripheral blood cells wa evaluated as a measure of the biologi impact of AZT. Because the predicte cellular effect of AZT in this combina tion would be termination of DN chain elongation secondary to AZ incorporation into replicating DNA, w hypothesized that strand breaks woul occur. A sample was obtained prior t administration of AZT and was used a an internal control for each patient. Th results, expressed as the percentage c double-stranded DNA based on a 100 sample obtained from cells from a no mal volunteer, are shown in figure DNA strand breaks were not observed a low doses of AZT. These data wer combined and demonstrate that 5-FU alone could not be responsible for th DNA strand breaks observed after AZ treatment at the higher dose levels. A AZT doses greater than or equal to 4. g/m² per day, a marked and significan increase in strand breaks occurred. I was not possible to demonstrate a dose dependent increase in strand break

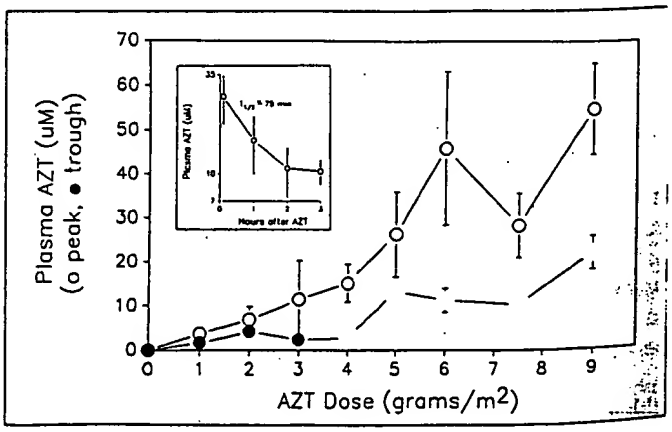

Figure 1. Peak and trough plasma levels of AZT. Samples were drawn at selected times, and AZT content was determined by high-pressure liquid-chromatography methods, as described in the text. Each point represents mean and SE of three to six determinations. Inset shows plasma clearance of AZT following peak plasma levels achieved after the last maximally tolerated oral dose of 7.5 g/m². Points represent mean and SEs in six patients.

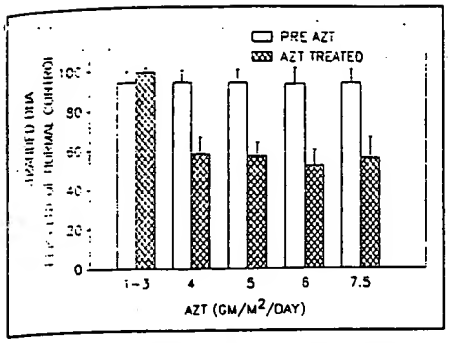

Figure 2. Percentage of double-stranded DNA observed in peripheral blood cells of the patients treated at each dose of AZT. Bar graphs represent means and SDs. Strand breaks in patients prior to starting AZT (PRE AZT) and during the last 24 hr of AZT administration (AZT TREATED) were compared with those in normal volunteers (100% double-stranded DNA). Data from 6 patients were averaged at 1–3 g/m² per day of AZT; subsequently, each bar graph represents data from 3–4 patients tested at each dosage level. At 4.0 g/m² per day of AZT and higher doses, 100 percent of patients tested developed strand breaks. Several patients were sampled below 4.0 g/m² per day of AZT as well as at or above this dose.

above that seen at the threshold level with the doses of AZT employed in this trial.

Discussion

In this report, we present the clinical, pharmacologic, and biochemical results of a phase I study of the novel chemotherapy combination of fixed-dose oral leucovorin and continuous-infusion 5-FU with escalating, high-dose, oral AZT. The notion that AZT can be an effective antineoplastic agent when used with drugs that alter pyrimidine metabolism is based on the exploitable differences between normal and neoplastic cells in their capacity to salvage thymidine and on the predicted metabolism of AZT (*9,16,17*). AZT has little intrinsic antineoplastic effect, since it is a poor substrate for mammalian DNA polymerase and must compete with thymidine for phosphorylation by TK (*17*). In the presence of agents that inhibit de novo dTMP production, it could be expected that AZT would effectively compete for phosphorylation by TK and be increasingly incorporated into DNA, causing premature chain termination (*12,18*). Moreover, observed differences in thymidine metabolism between tumor cells and normal tissues indicate that tumor cells can salvage thymidine more efficiently, although they cannot concentrate it from extracellular sources (*5,8*). The summation of these differences, combined with the potential increased specific activation of AZT under conditions of inhibition of de novo dTMP synthesis, leads to the hypothesis that high plasma levels of AZT, in combination with inhibitors of thymidylate synthase such as 5-FU, would have synergistic antitumor activity (*9–11*).

In vitro and in vivo trials that support this hypothesis have been performed. The in vitro combination of AZT and 5-FU exerts synergistic cytotoxicity against human tumor cell lines, in contrast to the effects seen with either agent alone. In studies of xenografted human tumors growing in nude mice, the combination of AZT and 5-FU also has produced substantial increases in antitumor effects, with marginal increases in myelotoxicity (*9*). Biochemical studies in mice have confirmed a relatively high degree of tumor tissue-specific toxicity compared with that of normal tissues (*9,10*). In addition to inhibiting thymidylate synthase as a means of increasing AZT antitumor effects, agents that increase thymidine requirements would also be expected to increase AZT efficacy. Support for this latter hypothesis was recently experimentally demonstrated in an in vitro model using cisplatin-induced DNA repair to increase thymidine requirements in the presence of AZT. Synergistic cytotoxicity was also observed in this model (*18*).

AZT, as used in human trials for human immunodeficiency virus (HIV-1), has shown little acute toxicity. Chronic anemia, megaloblastic marrow changes, leukopenia, muscle weakness, and nausea and vomiting have been observed. Oral absorption of the drug is excellent, with 50%–60% of the ingested dose absorbed and bioavailable (*17*). In the present trial, oral, high-dose AZT has been associated with significant and dose-limiting nausea and vomiting. Although this may have been a centrally mediated effect of the drug, it is possible that this is a local effect related to AZT concentration in the gut or mechanical factors associated with the large number of pills ingested. These later problems can be circumvented by employing intravenous AZT.

AZT induces DNA strand breaks in human colon tumor cell lines, and the degree of strand breakage correlates directly with in vitro cytotoxicity. Strand breaks are not observed with 5-FU but are enhanced by the combination of 5-FU and AZT and correlate directly with the synergistic cytotoxicity of the combination. Similar results have been observed in xenografted human tumors in nude mice. These results suggest that DNA strand breaks might serve as a marker of biologic effects of AZT in the presence of 5-FU (*10,11*). As AZT plasma levels in patients increased with increasing dose, peripheral blood cells developed DNA strand breaks at a threshold level of 4.0 g/m² per day. Peak and trough plasma concentrations at this level were 15 and 3 μM, respectively. This phenomenon is directly attributable to the increased AZT plasma levels and not to 5-FU or leucovorin, since it was not present at lower AZT doses in the presence of identical leucovorin and 5-FU therapy.

No further dose-dependent increase in strand breaks was observed. This result may reflect the observed lack of significant, dose-related increases in trough plasma levels above 10 μM. Alternatively, this result may be partly explained by the presence in the peripheral circulation of a small population of cells with a brief turnover time, such as short-lived polymorphonuclear cells, which might have suffered maximal DNA damage at the 4 g/m²-per-day dose during the restricted sampling period. In addition, a third parameter, related to DNA-repair capacity, may be present. This latter mechanism is supported by the lack of myelotoxicity seen in the patients. The extent of strand breaks in stem cell populations may be insufficient to overwhelm such DNA-repair mechanisms in cycling cells but may have been evident in the more differentiated, short-lived, nonreplicating peripheral blood cells. The failure to induce strand breaks may be overcome by sustained high plasma drug levels such as those obtained in murine models (*9–11*).

We conclude from this trial that oral AZT in combination with 5-FU and leucovorin can be given safely at 7.5 g/m² per day. At this dose, a biologic effect attributable to AZT is clearly evident. Although no tumor responses were seen in this small, heavily pretreated population, this study has demonstrated the feasibility and potential biologic effects of this combination. Further trials with intravenous AZT to achieve higher sustained plasma levels and circumvent the oral dose-limiting toxicity of nausea and vomiting are warranted.

References

(1) KEMENY N: Role of chemotherapy in the threatment of colorectal carcinoma. Semin Surg Oncol 3:190–214, 1987

(2) MORGAN RG: Leucovorin enhancement of the effects of the fluoropyrimidines on thymidylate synthase. Cancer 63:1008–1012, 1989

(3) BLEYER WA: New vistas for leucovorin in cancer chemotherapy. Cancer 63:995–1007, 1989

(4) POON MA, O'CONNELL MJ, MOERTEL CG, ET AL: Biochemical modulation of fluorouracil: Evidence of significant improvement of survival and quality of life in patients with advanced colorectal carcinoma. J Clin Oncol 7:1407–1418, 1989

(5) WEBER G, JAYARAM HN, PILLWEIN K, ET AL: Salvage pathways as targets of chemotherapy. Adv Enzyme Regul 26:335–352, 1987

(6) DENTON JE, LUI MS, AOKI T, ET AL: Enzymology of pyrimidine and carbohydrate metabolism in human colon carcinomas. Cancer Res 42: 1176–1183, 1982

(7) PATERSON ARP, KOLASSA N, CASS CE: Transport of nucleoside drugs in animal cells. Pharmacol Ther 12:515–536, 1981

(8) WOHLHUETER RM, MARZ R, PLAGEMANN PGW: Thymidine transport in cultured mammalian cells. Kinetic analysis, temperature dependence and specificity of the transport system. Biochim Biophys Acta 553:262–283, 1979

(9) BRUNETTI I, FALCONE A, CALABRESI P, ET AL: 5-Fluorouracil enhances azidothymidine cytotoxicity: In vitro, in vivo and biochemical studies. Cancer Res 50:4026–4031, 1990

(10) BRUNETTI I, FALCONE A, CALABRESI P, ET AL: Fluorouracil enhances azidothymidine antineoplastic activity and incorporation into tissue nucleic acids. Proc Am Assoc Cancer Res 31:2377, 1990

(11) DARNOWSKI JW, BRUNETTI I, POSNER MR, ET AL: Fluorouracil plus azidothymidine cytotoxicity in vitro: Relationship to cellular thymidine kinase activity. Proc Am Assoc Cancer Res 31:2361, 1990

(12) POSNER MR, BELLIVEAU JF, WEITBERG AB, ET AL: Continuous infusion cisplatin and bolus 5-fluorouracil in colorectal carcinoma. Cancer Treat Rep 71:975–977, 1987

(13) CALABRESI P, FALCONE A, ST. CLAIR MH, ET AL: Benzylacyclouridine reverses azidothymidine-induced marrow-suppression without impairment of anti-HIV activity. Blood. In press.

(14) BIRNBOIM HC, JEVCAK JJ: Fluorometric method for rapid detection of DNA strand breaks in human white blood cells produced by low doses of radiation. Cancer Res 41:1889–1892, 1981

(15) WEITBERG AB, CORVESE D: Hydroxy- and hydroperoxy-6,8,11,14-eicosatetraenoic acids induce DNA strand breaks in human lymphocytes. Carcinogenesis 10:1029–1031, 1989

(16) DARNOWSKI JW, HANDSCHUMACHER RE: Enhancement of fluorouracil therapy by th manipulation of tissue uridine pools. Pharmacol Ther 41:381–392, 1989

(17) YARCHOAN R, MITSUYA H, MYERS CE, ET AL: Clinical pharmacology of 3′-azido-2′3′-dideoxythymidine (zidovudine) and related dideoxynucleosides. N Engl J Med 321:726–738, 1989

(18) SCANLON KJ, KASHANI-SABET M, SOWERS LC: Overexpression of DNA replication and repair enzymes in cisplatin-resistant human colon carcinoma HCT8 cells and circumvention by azidothymidine. Cancer Commun 1:269–275, 1989

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Commentary
Telomerase as an anti-cancer target: current status and future prospects

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Introduction

The functional importance of the ends of chromosomes, telomeres, as major regulators of the cell's life and ultimate death was established many years ago (Blackburn, 1991). In human cells at birth, telomeres consist of ~15 kb of repeated TTAGGG DNA sequence of which ~100 bp is lost at every cell division due to the 'end-replication' problem. Eventually, if unchecked, progressive telomere erosion leads to cellular senescence. It is now clear that germline cells, proliferative stem cells and, significantly, most tumour cells maintain telomeres through activation of the enzyme telomerase, a ribonucleoprotein reverse transcriptase (Kim *et al.*, 1994). These findings were very quickly incorporated into theories of cellular immortalization, and undoubtedly have been the basis of the subsequent widely held view of telomerase as a highly selective anti-cancer target. The key linkage with tumorigenesis has been confirmed by a very large number of studies subsequent to the original ones (Kim *et al.*, 1994) which confirmed that 80–90% of a wide variety of human tumours show elevated levels of telomerase expression, specifically of the hTERT catalytic subunit, whereas telomerase is not detectable in normal somatic cells. Furthermore, clinical–laboratory translational studies have shown a correlation between high telomerase activity in tumours [e.g. neuroblastoma (Hiyama *et al.*, 1995) and breast cancer (Clark *et al.*, 1997)] and poor outcome (survival). Notably, early-stage neuroblastomas possess low telomerase activity whereas late-stage disease has higher levels. However, the precise role of telomerase in tumorigenesis has excited continuing controversy, as in turn has its validity as an anti-tumour target. We believe that the weight of evidence is now firmly indicative of telomerase being a key player in cellular proliferation and the initial events leading to malignancy, rather than being merely a bystander. Thus telomere length is seen to be an essential (though not necessarily the sole) signal for the unrestricted proliferation of immortalized cells. This view has led to a rapid increase in interest in telomerase as a potential anti-cancer target (Hamilton and Corey, 1996; Raymond *et al.*, 1996; Sharma *et al.*, 1997; Pitts and Corey, 1999). The various papers in this special issue of *Anti-Cancer Drug Design* reflect this recent upsurge in activity.

The cell and molecular biology of telomerase continues to increase in complexity. It was initially reported that even though there was a decline in telomere length in telomerase RNA-knockout mice, the mice did not appear to show any significant adverse effects over the first six generations (Blasco *et al.*, 1997). Furthermore, these telomerase-negative primary mouse cells could be immortalized, transformed by viral oncogenes, and were then tumorigenic in mice, suggesting that telomerase was not playing an essential role in the early stages of tumorigenesis. Subsequent studies on later generations of these mice, however, showed a variety of defects especially in highly proliferative organs (Lee *et al.*, 1998), which have resulted in a reappraisal of the role of telomerase and an affirmation that it is essential for cellular proliferation (Niida *et al.*, 1998). It is likely that the initial study did not take sufficient account of the very extended length of murine telomeres (30–40 kb) and the consequent need to examine later generations of the mice, as was subsequently done.

A variety of experimental approaches have more recently all confirmed the role of telomerase. Thus, normal human cells (retinal pigment epithelial cells and skin fibroblasts) have been transfected with the telomerase catalytic subunit gene, *hTERT*, with a resulting increase in life-span, telomere elongation and reduced senesecence, in striking contrast to telomerase-n gative control cells, which showed telomere shortening and entry into a senescent state (Bodnar *et al.*, 1998). Experiments with cells expressing hTERT carrying a mutant C-terminus has shown that these do not become

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immortalized and do not maintain telomere length, in contrast to cells expressing the wild-type gene (Counter *et al.*, 1998). Additional recent studies showed that, when a dominant-negative form of hTERT was introduced into various tumour cells, this resulted in complete inhibition of telomerase activity, reduction in telomere length, death of the cells and, moreover, prevented tumour formation in mice (R. Weinberg, personal communication). The rate of cellular arrest in each cell type was dependent upon initial telomere length. Very recent studies with mice doubly null with telomerase RNA and the tumour suppressor gene *INK4a* have broadly confirmed these findings, and have shown that loss of telomere function leads to impairment of tumour formation (Greenberg *et al.*, 1999).

There is increasing evidence that telomerase activation by itself is insufficient for immortalization, and that companion events such as inactivation of the retinoblastoma tumour suppressor pathway (Kiyono *et al.*, 1998) are required. Telomerase expression is directly up-regulated by the E6 viral (Greider, 1999) and c-MYC (J. Wang *et al.*, 1998; Greenberg *et al.*, 1999; Wu *et al.*, 1999) oncoproteins. Observations of telomerase expression not producing net telomere lengthening yet still resulting in extended cellular lifespan (Ouellette *et al.*, 1999; Zhu *et al.*, 1999) are actually representative of the situation in human tumours, since the presence of activated telomerase might be expected to result in increased telomere length compared with those in somatic cells. The fact that tumour telomeres are shortened yet with stabilized lengths (typically 2–7 kb) strongly suggests the importance of regulatory mechanisms, perhaps involving telomerase autoregulating itself via capping telomere ends, thereby enabling proliferation to continue even when telomeres are short (Zhu *et al.*, 1999). The roles of telomere-binding proteins such as TRF1 and the recently discovered tankyrase (Smith *et al.*, 1998) and TRF2 (Karlseder *et al.*, 1999) in these regulatory processes remain to be fully elucidated, although if shown to be acting in a true regulatory manner they could also be prime targets for potential therapeutic intervention. Indeed, in telomeres lacking TRF2, it has recently been shown that, in some cell types, p53- and ATM kinase-dependent apoptosis results rather than senescence (Karlseder *et al.*, 1999). A further complication is provided by increasing evidence for the existence of telomerase repressor sequences on chromosome 3p (Cuthbert *et al.*, 1999). Transfer of chromosome 3 into a human breast carcinoma cell line resulted in strong repression of telomerase and subsequent permanent growth arrest after 10–18 population d ublings.

Approaches to telomerase Inhibition

In spite of the strong association between telomerase expression and the malignant phenotype, the emerging cellular biological pr perties of telomeres and telomerase as outlined above have caused some to question their validity as an anticancer target. Commonly mentioned issues are (i) the possible requirement for prolonged inhibition in order to reduce telomeres to critical lengths. This would necessitate long-term clinical application after removal of the bulk of the tumour by surgery or cytotoxic chemotherapy, or use in a chemopreventative setting; (ii) the possibility of normal tissue toxicity in stem (T and B) and germ cells where telomerase is expressed; and (iii) the possible occurrence of drug resistance as some tumour cells have been shown to maintain telomeres by an alternative mechanism (Bryan *et al.*, 1997). However, there are also experimental data which provide counterarguments to each of the above; for example, rapid induction of apoptosis or loss of viability with dominant-negative hTERT or by targeting TRF2; telomere lengths in rapidly dividing tumour cells maybe critically short compared with slower-dividing normal cells. These issues remain speculative until such time that potent and selective inhibitors are discovered and thoroughly evaluated.

The characterization of the principal components of the enzyme as (i) the catalytic subunit hTERT with reverse transcriptase activity (Meyerson *et al.*, 1997); and (ii) an endogeneous RNA subunit containing the 11-base template sequence (nucleotides 46–56) on which telomeres are synthesized, hTR (Feng *et al.*, 1995) [for a recent review of telomerase structure and mechanism see O'Reilly *et al.* (O'Reilly *et al.*, 1999)], has clarified the various possibl options for inhibiting the key steps involved in telomere synthesis. A summary of various possible strategies is shown in Figure 1. Broadly, three options have been explored to date; active site small molecule inhibitors, oligonucleotides targeted against hTR or hTERT and telomere-directed agents, primarily directed at guanine (G)-quadruplex structures.

Telomerase inhibitors should reduce telomerase activity in cell extracts, ideally at submicromolar concentrati ns. Evaluation of activity has mostly been performed by procedures based on the highly sensitive PCR-based telomerase repeat amplification protocol (TRAP) assay. 'Direct' assays using

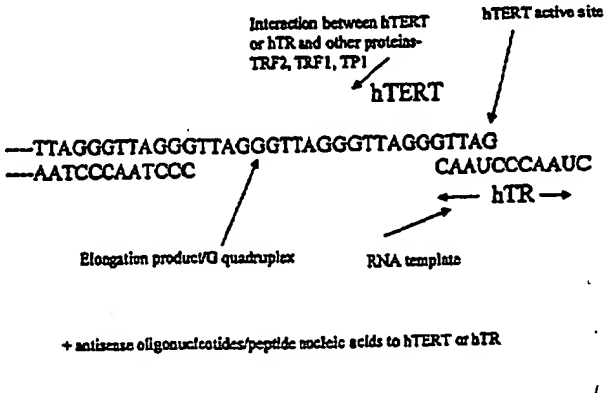

Figure 1

Strategies for inhibition of telomerase.

large amounts of partially purified enzyme have not been as widely used. These are traditionally time-consuming, much less sensitive than amplification-based methods and tend to give less reproducible results than the TRAP method. A variant using a sensitive chemiluminescent acridine ester oligonucleotide probe is claimed to be rapid and accurate (Lackey, 1998), even though it is 3–4 orders of magnitude less sensitive than the PCR-based methods. A recently developed bead-based direct assay using biotinylated primer (Sun *et al.*, 1998; Raymond *et al.*, 1999) has high sensitivity.

The classic model for telomere attrition stipulates that after ~20 rounds of cell doubling, cells will have critically short telomeres and will then enter the crisis state of non-proliferation, senescence. This was confirmed by initial antisense experiments directed at hTR in HeLa cells, where cells lost telomeric DNA and began to die after 23–26 doublings (Feng *et al.*, 1995). Accordingly, in order to validate the model, cell-based experiments with telomerase inhibitors require measurements of telomere length before and following exposure in addition to determining telomerase activity. The model predicts that long-term exposure of tumour cells to telomerase inhibitors (at concentrations which do not cause acute cytotoxicity) should induce telomere shortening and growth arrest, the rate being dependent upon initial telomere length. Chemically related molecules which do not inhibit the enzyme in cell-free extracts should not cause decreased cell proliferation or telomere shortening. In addition, an objective assessment of the onset of cellular senescence is necessary. Judgements have been made based on cellular morphological changes, and on the expression of senescence-associated β-galactosidase (X. Wang *et al.*, 1998). Telomere length estimation by means of fluorescence *in situ* hybridization (QFISH) has been successful in demonstrating length reduction in melanoma (K-1735) and breast cancer (MCF-7) cell lines (Multani *et al.*, 1998), using AZT for telomerase inhibition. Irreversible telomere shortening has also been demonstrated in HeLa cells using Southern blotting, following AZT treatment (Gomez *et al.*, 1998).

Active-site inhibitors

The reverse transcriptase activity of telomerase has unsurprisingly led to the appraisal of nucleotide-based molecules with established activity against viral RTs. Thus AZT has been shown in several studies to be active at the micromolar level (Melana *et al.*, 1998; Multani *et al.*, 1998), as have several other nucleotide triphosphates (Pai *et al.*, 1998).

A problem common to these nucleotides is their lack of specificity, in that they tend to be promiscuous in their ability to inhibit polymerases, not least *Taq* polymerase itself (the enzyme used in the amplification step of the TRAP assay). No specific active-site telomerase inhibitor has been reported to date in the open literature, although it is entirely possible that systematic screening of compound libraries has already resulted in such a compound being discovered. Screening of a 125 000 comp und library using a time-resolved fluorescence assay to detect PCR-amplified telomerase products has resulted in the identification f three isothiazolone compounds with IC_{50} values in the 130–140 nM range (Bare *et al.*, 1998), which may function by modifying cysteine residues at or close to the active site. Rational design of active-site telomerase inhibitors will have to await a high-resolution crystal-structure analysis of the catalytic domain, which is probably some way in the future. There are reports of telomerase inhibition by small molecules which do not appear to fit into any of the other categories discussed here [for example, that by catechins from tea (Naasani *et al.*, 1998), with reported ability to produce telomerase inhibition, telomere shortening and sensecence]. Whether these compounds are active-site inhibitors remains to be demonstrated. Reverse-transcriptase inhibition has not been observed by the fungal metabolite alterperylenol (Togashi *et al.*, 1998), which inhibits telomerase with an IC_{50} of 30 μM.

Oligonucleotides targeted against the RNA template

Kim *et al.* (1994) first showed that appropriate antisense oligonucleotides could effectively down-regulate telomerase-catalysed extension of telomeres, although it has been surprisingly difficult with such experiments to unequivocally demonstrate telomere shortening after a number of rounds of replication. Several more recent studies have reported anti-tumour effects with antisense oligonucleotides. Success has also been obtained in a cell-free system and in cultured carcinoma cells with a hammerhead ribozyme targeting the telomerase RNA template region (Yokoyama *et al.*, 1998), and with a 2′-*O*-methyl-modified rib zyme targeting telomerase in human glioma cell lysates (Wan *et al.*, 1998).

The oligonucleotide approach suffers from the problems of stability and uptake common to oligonucleotide therapeutics, although promising *in vitro* results have been obtained with a variety of backbone modifications which can at least partially counter nuclease susceptibility. PNA oligomers have been found to be especially potent inhibitors, typically with IC_{50} values in the nanomolar range. A systematic scanning f the 11-base RNA template sequence with a range of overlapping PNA oligomers (Hamilton *et al.*, 1997) showed the importance of bases in the alignment domain of the template site, as well as those in the elongation domain itself. Conjugation of short-length PNAs with cationic peptides linked at the equivalent of the PNA 5′-end have shown enhanced telomerase inhibition (Harrison *et al.*, 1999), with the best conjugate having an IC_{50} of 0.14 μM. 2′-*O*-Methylation of RNA ligomers is an effective stabilization strategy. Even though such oligomers have lower affinity for the RNA

template than analogous PNA ones, their telomerase inhibition is surprisingly sup rior (Pitts and Corey, 1998). The problem of PNA oligomers not being able to cross cell membranes has been addressed in studies using PNAs conjugated with peptides from the homeodomain f *Antennapedia*, which are effectively transported into mammalian cells (Simmons *et al.*, 1997). Simple lipid-mediated uptake of non-conjugated PNAs by DU145 cells has also been achieved (Hamilton *et al.*, 1999).

Problems of cellular delivery have been highlighted in a study with phosphorothioate-modified oligonucleotides, which effectively inhibited telomerase activity in cell lysate but were ineffective with intact cells (Duncan *et al.*, 1998). A further potential problem with phosphorothioate-modified oligonucleotides has been highlighted by the suggestion that they bind to the protein rather than the RNA component of telomerase (Matthes and Lehmann, 1999).

An interesting observation has been reported using an antisense strategy directed at telomerase RNA of human glioma cells (Kondo *et al.*, 1998). A 19-mer oligonucleotide linked to a 2',5'-oligoadenylate (2–5A) which activates the endoribonuclease, RNase L, induced a rapid (within 4 days) loss of cell viability with concomitant apoptosis. These results are contrary to the widely perceived need for an extensive lag phase (dependent upon telomere length) prior to the induction of cellular senescence.

Telomere-directed agents

The strategy of targeting telomerase indirectly, via the telomere primer strand, was originally devised for amido-anthraquinone-based telomerase inhibitors (Sun *et al.*, 1997). It was envisaged that these molecules would preferentially interact with telomeres when folded into four-strand G-quadruplex structures (Mergny and Hélène, 1998), which are known to inhibit the elongation steps catalysed by telomerase (Fletcher *et al.*, 1998; Zahler *et al.*, 1991). Evidence, albeit indirect, for this mechanism initially came from the observation that inhibition only occurred after 3–4 telomere repeats had been synthesized, consistent with the requirement of the human telomere repeat d(TTAGGG) for four repeats in order to fold into an intramolecular G-quadruplex structure (Sun *et al.*, 1997). More recent supportive evidence has come from experiments using 7-deazaGTP instead of GTP for primer incorporation (Han *et al.*, 1999b). The former cannot readily participate in G-quadruplexes and inhibition is decreased. Correlative evidence has also come from molecular modelling studies of series of inhibitors interacting with G-quadruplexes, where predicted affinities to them parallel telomerase inhibition abilities. A variety of planar aromatic molecules have now been shown to inhibit telomerase by this mechanism, including tetrapyridyl-substituted cationic porphyrins, a perylene derivative (Fedoroff *et al.*, 1998), acridines (Harrison *et al.*, 1999) and fluorenones (Perry *et al.*, 1999), as well as the four major regioisomers of bis-substituted amido-anthraquinones (Perry *et al.*, 1998a, 1998b).

The structural features necessary for activity and tel mere binding are (i) extended planar or n ar-planar electron-deficient chromophore; (ii) cationic charge, either on a side-chain(s) or on the chromophore itself; and (iii) if side-chains are present, then their lengths as well as the nature and size of the pendant end-groups are critical (Perry *et al.*, 1998a, 1998b). However, these features have not as yet been optimized, with the best inhibitors of this type having IC_{50} values in the 1–2 μM range, which is broadly comparable to their cytotoxicities. If such compounds are to be effective telomerase inhibitors *in vivo*, then their cytotoxic potencies must be low by comparison so that classic cytotoxic-mediated cell kill is at a minimum. These small molecule compounds have several advantages over oligonucleotide-based inhibitors, even though the latter can have superior activity in cell-free systems by several orders of magnitude. For example, pharmacophore-based inhibitors are more readily amenable to systemic administration, and are less likely to produce adverse immunological effects.

The mechanistic and structural details of these G4-stabilizing molecules are beginning to be explored. Of particular importance are kinetic factors, since the rate of formation of native G-quadruplexes is known to be very slo even in the presence of high concentrations of sodium or potassium ion. The perylene derivative PIPER has been found (Han *et al.*, 1999a) to accelerate this process some 100-fold, a result that is likely to have general significance for other G4 stabilizers. No crystal structure for a G-quadruplex–ligand complex is as yet available, although several plausible models based on NMR data have been produced, notably for complexes involving tetra-(*N*-methyl-pyridyl) porphyrin and PIPER (Wheelhouse *et al.*, 1998; Izbicka *et al.*, 1999). Although neither of these studies involves the human intramolecular G-quadruplex, both concur in showing that the planar ligands do not intercalate between adjacent G-quartets, presumably on account of their exceptionally slow dissociation kinetics. Instead, they are pseudo-intercalated/stacked at the ends of quadruplex structures.

It has not as yet been demonstrated that the telomerase inhibitory ability of these telomere-binding ligands can result in telomere shortening and senescence in cells. *In vitro* data on cationic porphyrins has shown that growth arrest (G_2–M cell cycle block) of human breast cancer cells can be achieved by exposure to subcytotoxic concentrations over 15 days (10 population doubling times) (Izbicka *et al.*, 1999). Telomerase activity in these MCF-7 cells decreased to a minimum level after only 4 days of exposure to porphyrin. H wever, n significant changes in telomere length were predicted to hav occurred (actual measurements were not reported) since

growth arrest was observed at much shorter times than would be required on the basis of rate of telomere loss even in cells with relatively short telomeres (>20 population doubling time). Similar observations have recently been made in our own laboratories in 21NT human breast cancer cells following long-term (14 days) exposure to non-cytotoxic concentrations of acridine-based G4-stabilizing molecules (S. Neidle and L.R. Kelland, unpublished observations). These G4-interactive agents await further study in tumour-bearing animals. Based on measurements of telomerase activity and limited telomere length, MCF-7 and MX1 breast cancer xenografts have recently been proposed as appropriate models (Raymond *et al.*, 1999).

Summary

As is common with a newly discovered cancer-associated gene/protein, there is a lag between the elucidation of its cellular and molecular biology and appropriate therapeutic intervention. Telomerase represents an interesting and promising anticancer drug target but poses a particular drug discovery challenge. It is unclear at present what is the optimum means of targeting this complex ribonucleoprotein and associated telomeric DNA and binding proteins: various strategies are actively being explored. Some recent data (e.g. 2–5A antisense against telomeric RNA, targeting TRF2, introduction of dominant-negative hTERT into cells) has raised doubts over the previously presumption of a requirement for prolonged enzyme inhibition with gradual telomere erosion, especially in tumour cells with relatively short telomeres. Highly potent and selective *in vivo* inhibitors are required to validate the target and address these critical issues.

References

Bare L.A., Trinh ion of a series of p time- resolved fluo ment *Research*, 43, 1

Blackburn E.H. (neres. *Nature*, 350, 56?.

Blasco M.A., Lee H.-W., Hande M.P., Samper E., Lansdorp P.M., DePinho R.A., Greider C.W. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell*, 91, 25.

Bodnar A.G., Quellette M., Frolkis M., Holt S.E., Chiu C.-P., Morin G.B., Harley C.B., Shay J.W., Lichtsteiner S., Wright W.E. (1998) Extension of life-span by introduction of telomerase int normal human cells. *Science*, 379, 349.

Bryan T.M., Englezou A., Dalla-Pozza L., Dunham M.A., Reddel R.R. (1997) Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nature Medicine*, 3, 1271.

Clark G.M., Osb rne C.K., Levitt D., Wu R., Kim N.W. (1997) Telomerase activity and survival of patients with Node-positive breast cancer. *Journal of the National Cancer Institute*, 89, 1874.

Counter C.M., Hahn W.C., Wei W., Caddle S.D., Beuersbergen R.I., Lansdorf P.M., Sedivy J.M., Weinberg R.A. (1998) Dissociation among *in vitro* telomerase activity, telomere maintenance, and cellular immortalization. *Cell Biology*, 95, 14723.

Cuthbert A.P., Bond J., Trott D.A., Gill S., Broni J., Marriott A., Khoudoli G., Parkinson E.K., Cooper C.S., Newb ld R.F. (1999) telomerase repressor sequences on chromosome 3 and induction of permanent growth arrest in human breast cancer cells. *Journal of the National Cancer Institute*, 91, 37.

Duncan V.E., Ajmani P.S., Hughes J.A. (1998) Cellular delivery is a major obstacle for oligodeoxy nucleotide inhibition of telomerase activity. *Anticancer Research*, 18, 4105.

Fedoroff O.Y., Salazar M., Han H., Chemeris V.V., Kerwin S.M., Hurley L.H. (1998) NMR-based model of a telomerase-inhibiting compound bound to G-quadruplex DNA. *Biochemistry*, 37, 12367.

Feng J., Funk W.D., Wang S.-S., Weinrich S.L., Avilion A.A., Chiu C.-P., Adams R.R., Chang E., Allsopp R.C., Yu J., Le S., West M.D., Harley C.B., Andrews W.H., Greider C.W., Villeponteau B. (1995) The RNA component of human telomerase. *Science*, 269, 1236.

Fletcher T.M., Sun D., Salazar M., Hurley L.H. (1998) Effect of secondary structure on human telomerase activity. *Biochemistry*, 37, 5536.

Gomez D.E., Tejera A.M., Olivero O.A. (1998) Irreversible telomere shortening by 3'-azido-2',3'-dideoxythymidine (AZT) treatment. *Biochemical and Biophysical Research Communications*, 246. 107.

Greenberg R.A., Chin L., Femino A., Lee K.-L., Gottlieb G.J., Singer R.H., Greider C.W., Depinho R.A. (1999) Short dysfunctional telomeres impair tumorigenesis in the $INK^{4a}\Delta^{2/3}$ cancer-prone mouse. *Cell*, 97, 515.

Greider C.W. (1999) Telomerase activation one step on the road to cancer? *Trends in Genetics*, 15, 109.

Hamilton S.F., Corey D.R. (1996) Telomerase: anti-cancer target or just a fascinating enzyme? *Chemistry and Biology*, 3, 863.

Hamilton S.E., Pitts A.E., Katipally R.R., Jia X., Rutter J.P., Davies B.A., Shay J.W., Wright W.E., Corey D.R. (1997) Identification of determinants for inhibitor binding within the RNA active site of human telomerase using PNA scanning. *Biochemistry*, 36, 11873.

Hamilton S.E., Simmons C.G., Kathiriya I.S., Corey D.R. (1999) Cellular delivery of peptide nucleic acids and inhibition of human telomerase. *Chemistry & Biology*, 6, 343.

Han H., Cliff C.L., Hurley L.H. (1999a) Accelerated assembly of G-quadruplex structures by a small molecule. *Biochemistry*, **38**, 6981.

Han H., Hurley L.H., Salazar M. (1999b) A DNA p lymerase stop assay for G-quadruplex-interactive compounds. *Nucleic Acids Research*, **27**, 537.

Harrison J.G., Frier C., Laurant R., Dennis R., Raney K.D., Balasubramanian S. (1999) Inhibition of human telomerase by PNA-cationic peptide conjugates. *Bioorganic & Medicinal Chemistry Letters*, **9**, 1273.

Hiyama E., Hiyama K., Yokoyama T., Matsuura Y., Piatyszek M.A., Shay J.W. (1995) Correlating telomerase activity levels with human neuroblastoma outcomes. *Nature Medicine*, **1**, 249.

Izbicka E., Wheelhouse R.T., Raymond R., Davidson K.K., Lawrence R.A., Sun D., Windle B.E., Hurley L.H., Von Hoff D.D. (1999) Effects of cationic phorphyrins as G-quadruplex interactive agents in human tumor cells. *Cancer Research*, **59**, 639.

Karlseder J., Broccoli D., Dai Y., Hardy S., de Lange T. (1999) P53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science*, **283**, 1321.

Kim N.W., Piatyszek M.A., Prowse K.R., Harley C.B., West M.D., Ho P.L.C., Coviello G.M., Wright W.E., Weinrich S.L., Shay J.W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011.

Kiyono T., Foster S.A., Koop J.I., McDougall J.K., Galloway D.A., Klingelhutz A.J. (1998) Both Rb/p16^{INK4a} inactivation and telomerase activity are required to immortalise human epithelial cells. *Nature*, **396**, 84.

Kondo S., Kondo Y., Li G., Silverman R.H., Cowell J.K. (1998) Targeted therapy of human malignant glioma in a mouse model by 2–5A antisense directed against telomerase RNA. *Oncogene*, **16**, 3323.

Lackey D.B. (1998) A homogeneous chemiluminescent assay for telomerase. *Analytical Biochemistry*, **263**, 57.

Lee H.-W., Blasco M.A., Gottlieb G.J., Horner J.W. II, Greider C.W., Depinho R.A. (1998) Essential role of mouse telomerase in highly proliferative organs. *Nature*, **392**, 569.

Matthes E., Lehmann C.H. (1999) Telomerase protein rather than its RNA is the target of phosphorothioate-modified oligonucleotides. *Nucleic Acids Research*, **27**, 1152.

Melana S.M., Holland J.F., Pogo B.G.-T. (1998) Inhibition of cell growth and telomerase activity of breast cancer cells *in vitro* by 3′-azido-3′-deoxythymidine. *Clinical Cancer Research*, **4**, 693.

Mergny J.L., Hélène C. (1998) G-quadruplex DNA: a target for drug design. *Nature Medicine*, **4**, 1366.

Meyerson M., Counter C.M., Eaton E.N., Ellisen L.W., Steiner P., Caddle S.D., Ziaugra L., Beijersbergen R.L., Davidoff M.J., Lie Q., Bacchetti S., Haber D.A., Weinberg R.A. (1997) *HEST2*, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell*, **90**, 785.

Multani A.S., Furlong C., Pathak S. (1998) Reduction of telomeric signals in murine melanoma and human breast cancer cell lines treated with 3′-azido-2′-3′-dideoxythymidine. *International Journal of Oncology*, **13**, 923.

Naasani I., Seimiya H., Tsuruo T. (1998) Telomerase inhibition, telomere shortening, and senescence of cancer cells by tea catechins. *Biochemical and Biophysical Research Communications*, **249**, 391.

Niida H., Matsumoto T., Satch H., Shiwa M., Tokutake Y., Foeli Y., Shinkai Y. (1998) Severe growth defect in mouse cells lacking the telomerase RNA component. *Nature Genetics*, **19**, 203.

O'Reilly M., Teichmann S.A., Rhodes D. (1999) Telomerases. *Current Opinion in Structural Biology*, **9**, 56.

Ouellette M.M., Aisner D.L., Savre-Train I., Wrights W.E., Shay J.W. (1999) Telomerase activity does not always imply telomere maintenance. *Biochemical and Biophysical Research Communications*, **254**, 795.

Pai R.B., Balakrishna P., Kukhanova M., Dutschman G.E. Guo X., Cheng Y.-C. (1998) Telomerase from human leukemia cells: properties and its interaction with deoxynucleoside analogues. *Cancer Research*, **58**, 1909.

Perry P.J., Gowan S.M., Reszka A.P., Polucci P., Jenkins T.C., Kelland L.R., Neidle S. (1998a) 1,4- and 2,6-disubstituted amidoanthracene-9,10-dione derivatives as inhibitors of human telomerase. *Journal of Medicinal Chemistry*, **4**, 3253.

Perry P.J., Reszka A.P., Wood A.A., Read M.A., Gowan S.M., Dosanjh H.S., Trent J.O., Jenkins T.C., Kelland L.R., Neidle S. (1998b) Human telomerase inhibition by regioisomeric disubstituted amidoanthracene-9,10-diones. *Journal of Medicinal Chemistry*, **41**, 4873.

Perry P.J., Read M.A., Davies R.T., Gowan S.M., Reszka A.P., Wood A.A., Kelland, L.R., Neidle S. (1999) 2,7-Disubstituted amidofluorenone derivatives as inhibitors of human telomerase. *Journal of Medicinal Chemistry*, **42**, 2679.

Pitts A.E., Corey D.R. (1998) Inhibition of human telomerase by 2′-*o*-methyl-RNA. *Proceedings of the National Academy of Science, USA*, **95**, 11549.

Pitts A.E., Corey D.R. (1999) The telomerase challenge—an unusual problem in drug discovery. *Drug Discovery Today*, **4**, 155.

Raymond E., Sun D., Chen S.-F., Windle B., Von Hoff D.D. (1996) Agents that target telomerase and telomeres. *Current Opinion in Biotechnology*, **7**, 583.

Raymond E., Sun D., Izbicka F., Mangold G., Silvas F., Windle B., Sharma S., Soda H., Laurence R., Davidson K., Von Hoff D.D. (1999) A human breast cancer model for the study of telomerase inhibitors based on a ne-

biotinylated-primer extension assay. *British Journal Cancer*, 80, 1332.

Sharma S., Raymond E., Soda S., Sun D., Hilsenbeck S.G., Sharma A., Izbicka E., Windle B., Von Hoff D.D. (1997) Preclinical and clinical strategies for development of telomerase and telomere inhibitors. *Annals of Oncology*, 8, 1063.

Simmons C.G., Pitts A.E., Mayfield L.D., Shay J.W., Corey D.R. (1997) Synthesis and membrane permeability of PNA-peptide conjugates. *Bioorganic & Medical Chemistry Letters*, 7, 3001.

Smith S., Giriat I., Schmitt A., de Lange T. (1998) Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science*, 282, 1484.

Sun D., Hurley L.H., Von Hoff D.D. (1998) Telomerase assay using biotinylated-primer extension and magnetic separation of the products. *Cancer Research Techniques*, 23, 1046.

Sun D., Thompson B., Cathers B.E., Salazar M., Kerwin S.M., Trent J.O., Jenkins T.C., Neidle S., Hurley L.H. (1997) Inhibition of human telomerase by a G-quadruplex-interactive compound. *Journal of Medicinal Chemistry*, 40, 2113.

Togashi K.-I., Kakeya H., Morishita M., Song Y.-X., Osada H. (1998) Inhibition of human telomerase activity by alterperylenol. *Oncology Research*, 10, 449.

Wan S.K.M., Fell P.L., Akhtar S. (1998) Synthetic 2'-*O*-Methyl-modified hammerhead ribozymes targeted to the RNA component of telomerase as sequence-specific inhibitors of telomerase activity. *Antisense, Nucleic Acid Drug Development*, 8, 309.

Wang J., Xie L.Y., Allan S., Beach D., Hannon G.J. (1998) Myc activates telomerase. *Genes & Development*, 12, 1769.

Wang X., Wong S.C.H., Pan J., Tsao S.W., Fung K.H.Y., Kwong D.L.W., Sham J.S.T., Nicholls J.M. (1998) Evidence of cisplatin-induced senescent-like growth arrest in nasopharyngeal carcinoma cells. *Cancer Research*, 58, 5019.

Wheelhouse R.T., Sun D., Han H., Han F.X., Hurley L.H. (1998) Cationic porphyrins as telomerase inhibitors: the interaction of tetra-(*N*-methyl-4-pyridyl)porphine with quadruplex DNA. *Journal of the American Chemical Society*, 120, 3261.

Wu K.-J., Grandori C., Amacker M., Simon-Vermot N., Polack A., Lingner J., Dalla-Favera R. (1999) Direct activation of *TERT* transcription by c-MYC. *Nature Genetics*, 21, 220.

Yokoyama Y., Takahashi Y., Shinohara A., Lian Z., Wan X., Niwa K., Tamaya T. (1998) Attenuation of telomerase activity by a hammerhead ribozyme targeting the template region of telomerase RNA in endometrial carcinoma cells. *Cancer Research*, 58, 5406.

Zahler A.M., Williamson J.R., Cech T.R., Prescott D.M. (1991) Inhibition of telomerase by G-quartet DNA structures. *Nature*, 350, 718.

Zhu J., Wang H., Bishop J.M., Blackburn E.H. (1999) Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. *Cell Biology*, 96, 3723.

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QUARTERLY

Minireview

Telomerase as a therapeutic target*

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Ribonucleoprotein telomerase is an enzyme that elongates telomeric DNA. In cells without detectable telomerase activity telomeres shorten with every cell generation and reaching critical length is a signal for cell death. Normal human somatic cells express undetectable, or low (bone marrow and peripheral leukocytes), telomerase activity. Reactivation of telomerase (immortalization) is probably necessary during development of a fully malignant cancer. Consequently, telomerase was proposed to be a therapeutic target for the cancer therapy. Potential results (including side-effects) of telomerase inhibition are being considered. After all, telomerase inhibition can be useful not only in the therapy, but also in cancer biology research, elucidating ageing and immortalization phenomena.

Eukaryotic chromosomes terminate with telomeres — a variable number of short species-specific telomeric repeats [1]. In mammals the sequence: TTAGGG (strand oriented 5′→3′ toward the chromosome terminus) is repeated. Telomeres appear to function in: chromosome stability — protection from DNA degradation, the end-to-end fusion and recombinations; chromosome positioning — attachment to the nuclear membrane; and replication. Telomeres are believed to form specialised structures, and interact with a number of associated proteins. Enzymes that elongate telomeric DNA (telomerases) are ribonucleoproteins [2]. They consist of an essential RNA and a few proteins. RNA contains a region complementary to telomeric repeats. It has been proved that these sequence serves as a template during synthesis of telomeric DNA. Therefore, telomerase is a specialised reverse transcriptase with the built-in template rather than a terminal transferase.

CELLULAR REPLICATIVE SENESCENCE

DNA-dependent DNA polymerases are unable to fully replicate ends of linear DNA (because they can synthesise DNA only in the 5′ → 3′ direction and require an RNA primer). Telomeres can be also lost by degradation by exonucleases. Thus, telomeres shorten in cells lacking telomere elongation activity. According to the telomere theory of cellular ageing, shortening of telomeres acts as a mitotic clock, responsible for cellular replicative senescence [3]. Normal human somatic cells express undetectable, or low (peripheral and bone marrow leukocytes) telomerase activity. Consequently, their telomeres shorten by 50–200 base-pairs with every cell generation. Reaching of critical length (after about eighty divisions for cells derived from a new-born child) is a signal for

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Abbreviations: TRAP, telomeric repeat amplification protocol.

finishing proliferation and afterwards cell death. The exact molecular mechanism of this proliferation crisis is not known. Both transcriptional activation — caused by changes in subtelomeric chromatin structure, and silencing of subtelomeric genes is possible. Silencing could result from changes in subtelomeric chromatin structure, or lack of enough space for transcriptional machinery between the point where a chromosome is anchored to the nuclear cage, and promoter/enhancer regions. Simple excision seems to be unlikely, since cells reaching the replicative limit (Hayflick's deadline) still have some telomeric DNA left.

There is some evidence that the so-called proliferational crisis consists of two stages. The first barrier, called the mortality stage 1 (M1), is associated with activation of p53 and retinoblastoma (RB) cycle-inhibiting genes, somehow induced by shortened telomeres. M1 can be bypassed by transformation with some oncogenes (traditionally called: immortalising oncogenes) for example SV-40 T-antigen. This is not, however, real immortalization. Transformed cells are still telomerase activity negative and show the telomeric decline. Eventually, they reach a second barrier called the mortality stage 2 (M2). M2 means that telomeres are physically too short to fulfil their functions any longer. Numerous chromosome aberrations, especially chromosome fusions, can be observed. Cells cannot divide; they will finally die unless telomerase is reactivated.

Unlike somatic cells, human germ-line cells express a high level of telomerase activity. This is necessary to ensure that telomeres will not shorten in next generations, leading to extinction of the species.

What we do not know is whether cellular replicative senescence is related to ageing of a multicellular organism. About eighty cell divisions, available for human cells, seem to be enough for a lifetime. Cells derived from very old individuals still can divide *in vitro*. What is more, rodents age although their somatic cells mostly express telomerase activity (however, their telomeres still generally shorten). On the other hand, premature shortening of telomeres was found in progeria (human disease associated with early ageing) [4]. However, accelerated accumulation of oxidised proteins was also found in progeria, suggesting rather oxidative than replicative senescence.

There is no doubt that cellular replicative senescence is a mechanism protecting against malignancy. One should remember that human cells are relatively resistant to transformation, both *in vitro* and *in vivo*. A single cell which starts to divide constantly (due to the activation of oncogenes and inactivation of antioncogenes) is a "mother" of malignancy. All cancer cells observed in the advanced disease are the progeny of the "mother" cell. Such a great number of divisions would be impossible without telomerase activity. Therefore, we can consider the reactivation of telomerase to be prerequisite for the development of malignancy in humans [5]. In the case of carcinogenesis *in vitro*, the telomerase recovery is probably responsible for overcoming the proliferation crisis of transformed cells (Fig. 1) [6, 7]. This is similar to carcinogenesis *in vivo*, since telomerase is not active in benign solid tumours (e.g. benign adenomas of colon, uterine fibroids and prostatic hyperplasia), and early leukaemias (e.g. acute myeloid leukaemia and chronic lymphocytic leukaemia), but its activity is detectable in solid malignant tumours and in advanced leukaemias [5, 8–10] (Fig. 1). When these findings are combined with the telomere theory of cellular ageing, we can see, that the step of immortalization (induced by the reactivation of telomerase, probably due to a mutation of its repressor) is necessary for the development of malignancy. This is strongly supported by the fact that immortalised human cell lines, or cell lines derived from rodents are much more susceptible to transformation *in vitro* [11].

There are, of course, facts which cannot be simply explained and seem to be exceptions from this attractive comprehensive theory. There are rare examples of malignant tumours without telomerase activity. It may be that cells of these cancers are not really immortal, but are able to spread and be harmful within their normal replicative life-span. It is also possible that these cells use another mechanism to elongate their telomeres. They could, for example, do it through the recombination-based pathway similar to that observed in yeast cells.

It is generally difficult to establish cultures of cancer cells *in vitro*. Why is it so if these cells are immortal? Early studies failed to demonstrat telomerase activity in fresh cancer samples. Now, with the new PCR-based assay: the telomeric repeat amplification protocol (TRAP),

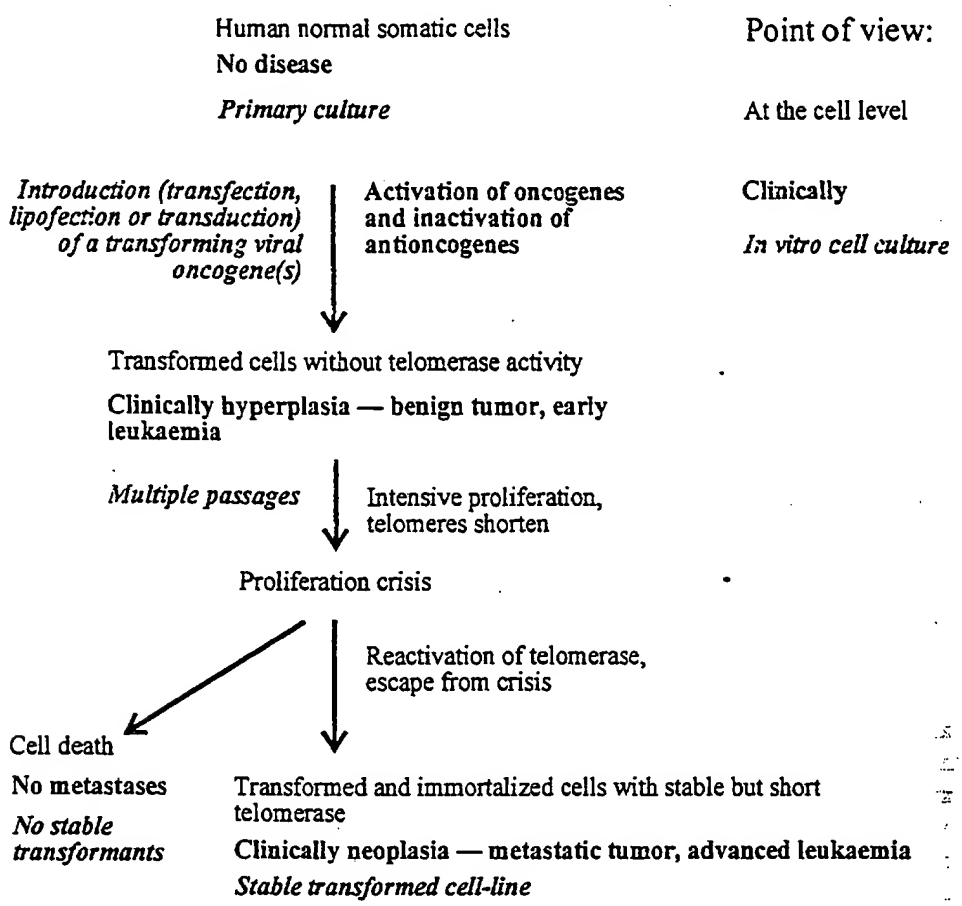

Fig. 1. New conception of carcinogenesis (in vivo and in vitro).

telomerase activity in about 85% of malignant cancers can be detected [5]. One could suppose that the new assay is too sensitive, and will detect extremely low levels of telomerase activity, too small to play a physiological function. The published results indicate that this is not true, but one cannot be sure, until the new assay is standardised and previous results are confirmed.

It was also proposed that telomerase is somehow turned off after the cell isolation. This hypothesis assumes that the phenomenon of telomerase reactivation requires unique environmental conditions, which cannot be maintained in the cell culture.

Latest data show that low telomerase activity can be also detected in bone marrow and peripheral blood leukocytes [9]. This suggests that somatic expression of the enzyme may be wider than was previously thought. It is not unlikely that telomerase activities in other somatic tissues are so low, that they cannot be detected even by the sensitive TRAP assay. Alternatively, bone marrow and peripheral blood leukocytes may be unique exceptions, and other tissues may not have telomerase at all (or more precisely nearly at all — we should not forget illegitimate transcription).

ANTI-TELOMERASE TREATMENT

Investigations focusing on the problem of telomerase activity in malignant cells are, of course, very important for understanding of the biology of cancer, but they could have an impact on therapy of malignancy, too. There are several currently ongoing clinical trials utilising strategies which are called "cancer repair" [12]. These protocols are based on intervention aimed at correcting the proliferation regulating mechanisms damaged by mutations which took place during carcinogenesis. So far, telomerase has not been usually considered to be a therapeutic target. Instead, delivering the wild-type copy of p53 antioncogene to malig-

nant cells [13, 14] (utilising the gene therapy technique) or silencing of the over-expressed oncogenes [15–17] (utilising the gene therapy, exogenous antisense oligonucleotides or conventional pharmacological inhibitors) are popular ideas. The immortalising enzyme — telomerase — is not strictly a product of oncogene, but arresting of its activity in malignant cells could cause proliferation crisis and afterwards their death.

One can argue that even if such a therapy worked, its action would be very slow, and patients would probably die before telomeres of their malignant cells would shorten to a critical length. Such a scenario is not unlikely, but there are facts which seem to be promising. Firstly, there is a negative correlation between telomerase activity and the average telomere length in transformed cells [8, 9]. This means that the telomerase activity positive transformed cells have shorter telomeres that normal cells (derived from the same tissue, and the same donor, or donor of the same age). During the early period of development of malignancy (benign tumour, early leukaemias) transformed cells divide losing telomeric DNA. When telomeres are critically short the proliferation crisis takes place. The crisis positively selects cells with reactivated telomerase. These cells are both transformed and immortalised. They survive, and can develop into malignancy. Their telomeres are stabilised, but still very short [18, 19]. An increased rate of the chromosome fusion, observed in many malignant cells, is probably the simple consequence of poor protection of chromosomes by these short telomeres. All these facts suggest that the main function of telomerase is the prevention of shortening rather than the intensive elongation of telomeres. Thus, inhibition of telomerase activity should quickly lead to reaching the proliferation limit (Hayflick's deadline).

In accordance with the latest results, telomerase activity is detectable not only in germ-line cells, but also (though it is much lower) in bone marrow and peripheral leukocytes [9]. So far, these are the only adult tissues in which telomerase has been detected. Thus, systemic anti-telomerase therapy could act as a very selective treatment for malignant tumours. Possible sterility could be probably an acceptable side-effect of therapy against advanced disease. The survival of germ-line cells with shorter telomeres would be worse, because in the next generations even faster ageing might take place. At present, it is not possible to predict potential toxic effects on leukocytes and their precursors, because both biological significance and the identity of the telomerase activity positive cells is unclear. Despite the presence of telomerase activity, telomeres in bone marrow and peripheral blood leukocytes still shorten. This was, however, shown by an assay measuring an average telomere length [9]. Thus, it could be assumed that a small subpopulation of these cells express high levels of telomerase — and do not show the telomeric decline, whereas other subpopulations express no telomerase activity — and their telomeres shorten. If so, the identity of telomerase positive subpopulation would be of great interest.

On the other hand, it is possible that all leukocytes and their precursors have some telomerase activity, but their telomeres shorten despite this activity. The rate of this shortening could be, however, slightly diminished by low level of telomerase activity. In fact, telomere loss rates vary among different cell types [8]. This could suggest that regulation of telomerase activity and telomere length is more complex than previously thought. Telomerase may be controlled not only at the level of RNA or protein expression, but also at the level of enzymatic activity. Furthermore, function of this ribonucleoprotein may not only be telomere elongation, but also maintaining the correct structure of telomeres (through incorporation into the telomeric nucleoprotein complex).

All these facts and hypothesis point to the need for great caution in the process of interpreting experimental data. Different parameters: telomere length, telomerase activity, the presence of telomerase RNA or the protein subunit, do not necessary correlate with each another. An average result will not ensure that there is no subpopulation of cells (for example stem cells) with entirely different characteristics. However, we know that most primary bone marrow stem cells (CD34+) lose their telomeric DNA during *in vitro* culturing [20]. This suggests that the telomerase activity positive cells may belong to a more differentiated compartment. This is optimistic, because toxicity against differentiated leukocyte precursors or peripheral leukocytes, even if it occurred, would not probably be life-threatening.

HOW CAN TELOMERASE BE SELECTIVELY INHIBITED?

Telomerase is a ribonucleoprotein. Its own integral RNA is used as a template for the synthesis of the DNA repeat motif characteristic of the species. Recently, a group of scientists from Cold Spring Harbour Laboratory and Geron Corporation, has cloned telomerase RNA [21]. They also proved that the introduction of a gene encoding antisense RNA targeted to telomerase RNA component into the HeLa immortalised cell-line, leads to the proliferation crisis, and cell death. Thus, antisense strategy seems to be very attractive means of telomerase inhibition.

It is hard to decide which of antisense strategies would be the best. If exogenous simple antisense strategy (deoxyoligonucleotides or their analogues) is used, the effect of inhibition will be the overall result of: 1) competition with the 3'-ended short single-stranded "overhang" at the end of telomere for binding to telomerase RNA in the holoenzyme; 2) competition with the telomerase protein component for binding to newly synthesized telomerase RNA; 3) degradation of the formed RNA:DNA dimers by RNase H. Since RNase H cannot degrade RNA:RNA dimers, mechanism 3) will not take place if we choose the gene therapy technique.

Gene therapy offers the exceptional possibility of the targeted delivery and transcription of a gene encoding antisense RNA (or other anti-telomerase genes). This could completely eliminate the potential toxicity to germ-line cells and leukocytes. Such a gene transfer would be, however, very difficult. Because the bystander-killing effect is rather unexpected (as for all "cancer repair" strategies), a 100% efficiency of transfer seems to be necessary. This would not be possible without new vector systems (e.g. a new generation of liposomes connecting high transfer efficiency of cationic liposomes with good pharmacokinetics and the targeted delivery to cancer cells of stealth liposomes [22]). When new gene transfer methods are worked out, anti-telomerase therapeutic genes could be very useful in systemic gene therapy for advanced malignancy. To enable intensive gene transfer, the targeted delivery *via* the ligand-receptor interaction should be probably excluded. The targeted transcription (use of an appropriate promoter) will be probably sufficient, since even non-targeted anti-telomerase treatment seems to be able to act very selectively against cancer cells.

Ribozyme antisense strategy (utilising exogenous or gene-derived ribozymes) is usually more effective than simple antisense strategy because ribozymes possess enzyme kinetics [23, 24]. However, there is evidence that RNA undergoing complex association with proteins may not form optimal structure for the ribozyme cleavage. For example, simple antisense strategy was more active than ribozyme strategy against RNA of a small nuclear ribonucleoprotein — U7 [25].

Antisense strategy could be targeted not only to the RNA component of the telomerase protein, but also to mRNA of the protein subunit. These two approaches are quite similar. Which of them would be more effective could depend on which factor (RNA or protein) exists in smaller quantities in the cell, and therefore limits the activity of the enzyme. Recently, it has been found that telomerase RNA is present in many human tissues which show no detectable telomerase activity [21]. This suggests that the protein component is more rigorously regulated than telomerase RNA. On the other hand, the antisense strategy targeted to the RNA component could not only prevent formation of the holoenzyme, but also inhibit the activity of created telomerase molecules. This inhibition might be very effective, since antisense oligonucleotide would compete with only 92 telomeres per cell.

The construction of any antisense molecule targeted to mRNA of the protein telomerase component requires cloning of the gene encoding this protein, which has not been done so far.

There are also other theoretically possible methods requiring the gene transfer; examples are: intracellular single-chain antibodies against the telomerase protein; transdominant mutants of the telomerase protein which can bind template, and form ribonucleoprotein without telomerase activity; "RNA decoys" similar to RNA template in that they can bind telomerase protein, but the created dimer will be inactive. These three strategies are modifications of the procedures known from gene therapy for the HIV infection, when a therapy target is to neu-

tralize some elements of the virus [26, 27]. However, they are highly experimental, and there is little experience with their use.

It is also very likely that an effective system of telomerase inhibition could be worked out in the field of "conventional" pharmacotherapy. The goal is to find selective inhibitors of the enzyme. They could be analogues of deoxynucleotides, with high affinity to telomerase, but low to DNA dependent DNA polymerases, working as terminating agents (they might stop elongation by lack of ability to create a bond with the next deoxynucleotide). The application of non-deoxynucleotide selective inhibitors is also possible. Looking for inhibitors of telomerase should include screening and drug designing. This would strongly resemble searching for inhibitors of HIV reverse transcriptase. After all, telomerase is a kind of reverse transcriptase with the built-in template.

The well known HIV reverse transcriptase inhibitor — AZT, has already been examined for its effect on the *Tetrahymena* telomerase. It was found that AZT induces fast telomere shortening in vegetatively growing *Tetrahymena* [28].

RODENT MODEL

We have a great many information about the telomerase regulation in mice tissues [29, 30]. These findings are amazing. In fact, most mouse tissues possess telomerase activity. The levels of this activity can differ a lot. Consequently, telomere length is similar in all tissues of a newborn mouse, but different in tissues of an adult mouse. In primary cell cultures of mouse fibroblasts telomerase activity is undetectable, and telomere length decreases with cell divisions. In contrast, after a frequent spontaneous immortalization, remarkable telomerase activity is present and telomeres maintain a stable length.

It was proposed that to protect them against malignancy, long-lived species need more stringent control over cell proliferation than short-lived species. This is not, however, self-evident. One could argue that long-lived species need more cell doublings — thus, should have active telomerase.

In order to explain this complicated issue other species should be investigated as well as it should be determined whether the observed telomerase activity is due to telomerase expression in all cells, or only in a limited subset of cells.

Due to the permanent telomerase expressioı in many somatic tissues, rodents — the most frequently used model in cancer research, apparently does not seem to be useful in this particular situation. However, some mouse tissues (for example skin) do not possess telomerase activity. Thus, an interesting proposal to use the mouse skin multistage chemical carcinogenesis model for such investigations, has been recently put forward [31].

CONCLUSIONS

The main purpose of this review is to attract attention to telomerase as a potential therapeutic target. Of course, the anti-cancer effect of inhibiting telomerase is highly theoretical. All the same, even if this assumption is false, studying the results of neutralizing telomerase should give us new knowledge about the cancer biology, ageing and the phenomenon of immortalization. Furthermore, telomerase might serve as the nearly universal malignancy marker in the cancer diagnostics. Finally, anti-telomerase treatment can be directed against fungi and protozoa. These eukaryotic unicellular organisms are (in the biological meaning) immortal and they constantly express telomerases [32–34]. Their telomeres and telomerases differ from human ones. Therefore, theoretically methods for selective inhibition of their telomerase activities could be found.

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REFERENCES

1. Blackburn, E.H. (1991) Structure and function of telomeres. *Nature (London)* 350, 569–573.
2. Blackburn, E.H. (1992) Telomerases. *Annu. Rev. Biochem.* 61, 113–129.
3. Harley, C.B., Vaziri, H., Counter, C.M. & Allsopp, R.C. (1992) The telomere hypothesis of cellular ageing. *Exp. Gerontol.* 27, 375–382.
4. Allsopp, R.C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E.V., Futher, A.B., Greide,

C.W. & Harley, C.B. (1992) Telomere lenghtening predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 89, 10114–10118.

5. Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L. & Shay, J.W. (1994) Specific associations of human telomerase activity with immortal cells and cancer. *Science* 266, 2011–2015.
6. Giraroli, A.J., Jensen, F.C. & Koprowski, H. (1969) SV40-induced transformation of human diploid cells: Crisis and recovery. *Int. J. Natl. Cancer Inst.* 42, 867–870.
7. Counter, C.M., Botelho, F., Harley, C.B. & Bacchetti, S. (1994) Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein-Barr virus transformed human B lymphocytes. *J. Virol.* 68, 3410–3414.
8. Steel, M. (1995) Telomerase that shapes our ends. *Lancet* 345, 935–936.
9. Counter, M.C., Gupta, J., Harley, C.B., Leber, B. & Bacchetti, S. (1995) Telomerase activity in normal leukocytes and in hematologic malignancies. *Blood* 85, 2315–2320.
10. Schwartz, H.S., Juliao, S.F. & Sciadini, M.F. (1995) Telomerase activity and oncogenesis in giant cell tumor of bone. *Cancer* 75, 1094–1098.
11. Blasco, M.A., Funk, W., Villeponteau, B. & Greider, C.W. (1995) Functional characterization and developmental regulation of mouse telomerase RNA. *Science* 268, 1267–1270.
12. Huminiecki, Ł. (1995) Gene therapy — vectors and strategies. *Adv. Biochem.* 4, 220–230.
13. Fujiwara, T., Grim, E.A., Mukhopadhyay, T., Cai, D.W., Owen-Schaub, L.B. & Roth, J.A. (1993) A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. *Cancer Res.* 53, 4129–4133.
14. Zhang, Y., Mukhopartyay, T., Donehower, L.A., Georges, R.N. & Roth, J.A. (1993) Retroviral vector-mediated transduction of *K-ras* antisense RNA into human lung cancer cells inhibits expression of the malignant phenotype. *Hum. Gene. Ther.* 4, 451–460.
15. Ratajczak, M.Z., Kant, J.A. & Luger, S.M., Hijiya, N., Zhang, J., Zon, G. & Gewirtz, M. (1992) *In vivo* treatment of human leukaemia in a *scid* mouse model with c-myb antisense oligodeoxynucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 89, 11823–11827.
16. Skorski, T., Nieborowska-Skorska, M. & Nicolaides, N.C. (1994) Suppression of Philadelphia 1 leukaemia cell growth in mice by BCR-ABL antisense oligonucletide. *Proc. Natl. Acad. Sci. U.S.A.* 91, 4504–4508.
17. Lange, W., Cantin, E.M., Finke, J. & Dolken, G. (1993) *In vitro* and *in vivo* effects of synthetic ribozymes targeted against BCR/ABL mRNA. *Leukemia* 11, 1786–1794.
18. Counter, C.M. (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* 11, 1921–1929.
19. Shay, J.W., Wright, W.E. & Brasiskite, D. (1993) E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. *Oncogene* 8, 1407–1413.
20. Vaziri, H., Dragowska, W. & Allsopp, R.C. (1994) Evidence for a mitotic clock in human hematopoietic stem cells, loss of telomeric DNA with age. *Proc. Natl. Acad. Sci. U.S.A.* 91, 9857–9860.
21. Feng, J., Funk, W.D., Wang, S.S., Weinrich, S.L., Avilion, A.A., Chiv, C.P., Adams, R.R., Chang, E., Allsopp, R.C. & Yu, J. (1995) The RNA component of human telomerase. *Science* 269, 1236–1240.
22. Allen, T.M. (1994) Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *Trends Pharmacol. Sci.* 15, 215–220.
23. Kashani-Sabet, M. & Scanlon, J.K. (1995) Application of ribozymes to cancer therapy. *Cancer Gene Ther.* 2, 213–223.
24. Mercola, D. & Cohen, J.S. (1995) Antisense approaches to cancer therapy. *Cancer Gene Ther.* 2, 47–59.
25. Cotten, M., Schaffner, G. & Birnstiel, M.L. (1989) Ribozyme, antisense RNA, antisense DNA inhibition of U7 small nuclear ribonucleoprotein-mediated histone pre-mRNA processing *in vitro*. *Mol. Cell. Biol.* 9, 4479–4487.
26. Bridges, S.H. & Sarver, N. (1995) Gene therapy and immune restoration for HIV disease. *Lancet* 345, 427–432.
27. Lever, A.M.L. (1995) Gene therapy for HIV infection. *Brit. Med. Bull.* 51, 149–166.
28. Kipling, D. (1995) Telomerase: Immortalising enzyme or oncogene. *Nature Genetics* 9, 104–106.
29. Prowse, K.R. & Greider, C.W. (1995) Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4818–4822.
30. Morin, G.B. (1995) Is telomerase a universal cancer target? *J. Natl. Canc. Inst.* 87, 859–862.
31. Bednarek, A., Budunova, I., Slaga, T.J. & Aldaz, C.M. (1995) Increased telomerase activity in

mouse skin premalignant progression. *Cancer Res.* 55, 4566–4569.

32. Bhattacharya, A. & Blackburn, E.H. (1995) Architecture of telomerase RNA. *EMBO J.* 13, 5721–5731.
33. McCormick-Graham, M. & Romero, D.P. (1995) Ciliate telomerase RNA structural features. *Nucleic Acids Res.* 23, 1091–1097.
34. Greider, C.W. (1990) Telomeres, telomerase and senescence. *Bio-Essays* 12, 363–369.

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Cytotoxic Interactions of 5-Fluorouracil and Nucleoside Analogues *in Vitro*

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Abstract. *The cytotoxic interaction of combined 5-fluorouracil (5-FU) with different nucleoside analogues was investigated in vitro on a colon (WiDr) and a breast (MCF-7) cancer cell line. Azidothymidine (AZT), 3'-deoxy-2',3'-didehydrothymidine (D4T), 5-iododeoxyuridine (IdUrd) and 2',3'-dideoxycytidine (DDC) were tested at different concentrations (5-600 μM) as modulators of 5-FU. The experimental endpoints were cellular viability and cell cycle distribution. The combination of 5-FU and AZT or D4T yielded supra-additive cytotoxic effects in both cell lines at all concentrations. On WiDr, IdUrd at high concentrations of 50 and 100 μM showed a supra-additive effect whereas at low concentrations (5, 10 and 20 μM) the effect was antagonistic. 5-FU combined with IdUrd produced a synergistic effect on MCF-7 cells at all concentrations. DDC antagonised the toxic effect of 5-FU on the WiDr cell line. In WiDr cells, a significant increase in the overall S-phase was observed 48 and 72 hours after exposure to D4T, AZT and DDC at the low concentration of 10 μM. On the contrary, this accumulation in S-phase was not present in MCF-7 cells. The combined effect of 5-FU and nucleoside analogues in vitro is dependent on the type and concentration of nucleosides and the cell-line tested. AZT, D4T and IdUrd are more likely to be subjected to more intensive in vitro and in vivo research as far as modulation of 5-FU toxicity is concerned.*

5-Fluorouracil (5-FU) is commonly used in the treatment of colon and breast cancer. Single-agent chemotherapy with 5-FU in these tumors results in response rates of 10-20%, but complete remissions are rare. In order to enhance the cytotoxic effect of 5-FU, several new treatment strategies have been developed [1-3]. Recently, it has been demonstrated that some nucleoside analogues such as AZT and IdUrd, both thymidine analogues widely used respectively in AIDS and in oncology, are synergistically cytotoxic with 5-FU or MTX in human colon cancer cells [4-9]. Scanlon and Szekeres *et al* have further reported that AZT can restore sensitivity to cisplatin and 5-FU *in vitro* in cells resistant to these agents [10,11]. Screening of nucleoside analogues as cytotoxic modulators of thymidylate synthase (TS) inhibitors remains an interesting issue. AZT and IdUrd as cytotoxic drugs combined or not to 5-FU are currently undergoing clinical evaluation [12-15]. D4T and DDC are clinically useful in the treatment of AIDS and related complexes [16,17]. Both compounds have not been previously reported as potential anticancer agents. In the present study, our intention was to evaluate the different nucleoside analogues (D4T, AZT, IdUrd and DDC) as potential modulators of the cytotoxic effect of 5-FU *in vitro* on human colon and breast cancer cell lines. From these experiments, D4T, AZT and IdUrd emerge as interesting drugs to be tested in combination with 5-FU *in vivo*.

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Key Words: Nucleoside analogues, 5-fluorouracil, chemosensitizer.

Materials and Methods

Chemicals. AZT and IdUrd were provided by the Wellcome Foundation LTD (London, England). D4T by Bristol-Myers, and DDC by Roche-Pharma. Dimethyl sulfoxide (DMSO), 5-FU, propidium iodide and 3-(4,5,-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemie (Buchs, Switzerland). MTT was dissolved at a concentration of 5 mg/ml in RPMI- 1640 medium without phenol red, filtered and frozen for stockage. 5-FU was initially dissolved in phosphate-buffered saline at concentration of 2 mM and stored at -20°C.

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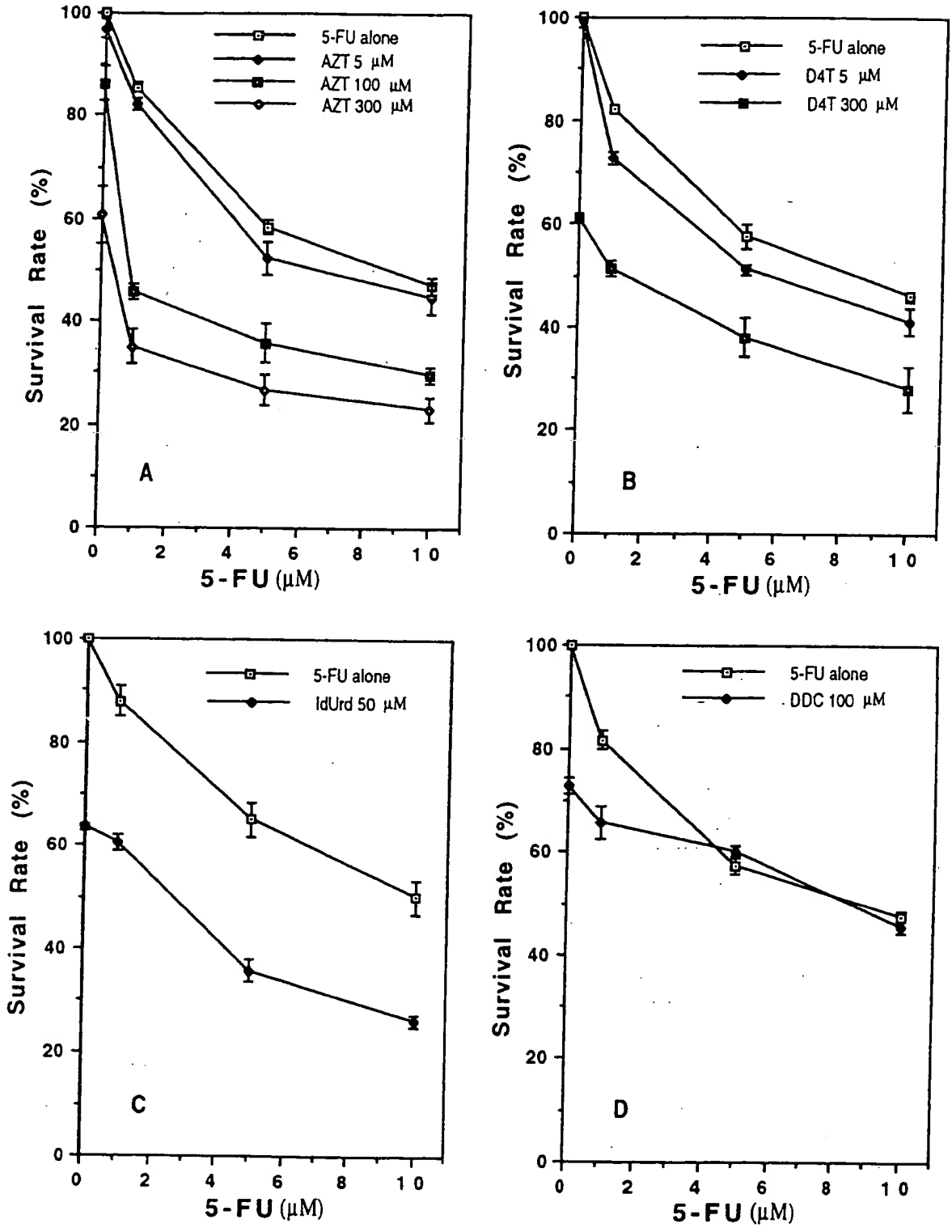

Figure 1. *Cytotoxicity of 5-FU as determined using a 3-day MTT assay in the absence or in the presence of the different concentrations of AZT (A), D4T (B), IdUrd (C) or DDC (D) on WiDr. Results are the mean ± SE of at least 3 independent experiments.*

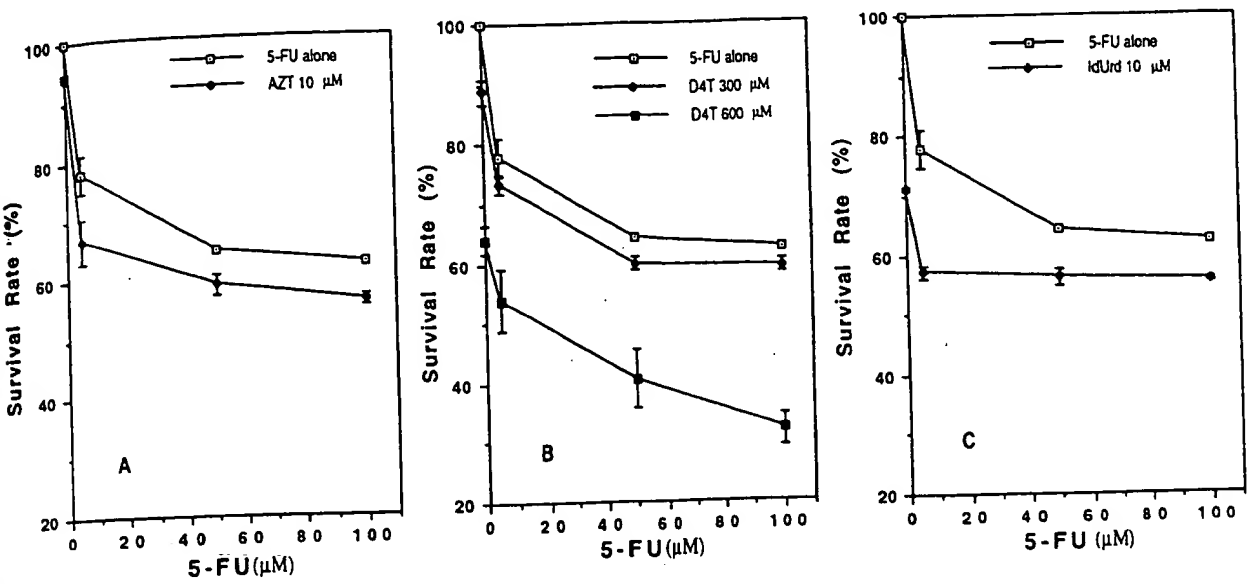

Figure 2. *Cytotoxicity of 5-FU as determined using a 3-day MTT assay in the absence or in the presence of the different concentrations of AZT (A), D4T (B) or IdUrd (C) on MCF-7. Results are the mean ± SE of at least 3 independent experiments.*

Cell culture. The human colorectal cancer cell line WiDr and breast cancer cell line MCF-7 were cultured at 37°C in 5% CO_2 and 95% air humidified atmosphere in Minimum Essential Medium with 10% fetal calf serum, 1 % non-essential amino acids and 2 mM L-glutamine and passed twice weekly. Under these conditions, the doubling time of the cells growing exponentially was approximately 23 hours for WiDr and 30 hours for MCF-7.

Cytotoxicity assay. A survival study was performed using a MTT assay as described by Denizot *et al* with some modifications [18]. One hundred microliters of cell suspension and 100 µl of diluted drug solution were mixed *per* well in a Falcon Primaria 96-wellplate. The cell concentration was 4200 cells/well for WiDr and 5100 cells/well for MCF-7. The plates were incubated for 72 hours. At this time point, both cell lines showed a lack of confluent growth, though cells should have divided at least two times. The culture-medium was removed, and 100µl MTT solution was added to each well. Following an additional 4 hours incubation, MTT was removed and 200µl *per* well of DMSO was added to dissolve the formazan crystals. The 96 well plate was shaken on a microplate shaker (EAS 2/4, SLT) for 5 minutes, and thereafter the optical densities (OD) of each well were read at 570 nm with the reference wavelength set at 690 nm using a SLT SPECTRA II (Tecan AG, Hombrechtikon, Switzerland) plate recorder.

The cell survival was calculated as the fraction of cell alive relative to control for each point:

cell survival (%) = (mean OD in the treated wells/mean OD in the control wells) × 100.

The IC_{50} (Inhibitory Concentration 50%) values were defined as the drug concentrations inducing a 50% reduction in the OD as compared to the untreated cells. The cytotoxic activity of the drug combination was evaluated by isobologram analysis [19].

D4T

Cell cycle analysis. Aliquots of WiDr and MCF-7 Cells in exponential growth were exposed to 10 µM AZT, D4T or DDC under standard culture conditions for 24, 48 or 72 hours. Thirty minutes prior to harvesting, bromodeoxyuridine (BrdUrd) was added at the final concentration of 10 µM. The cells were trypsinized and fixed in 70% alcohol. The cells were stained for BrdUrd and DNA contents with slight modifications of the classical method of Begg [20] and maintained in the dark at 4°C for at least 24 hours until analysis.

The cells were analysed in a FACSCAN (Becton Dickinson) flow cytofluorometer. Green (BrdUrd incorporation) and Red (DNA content) fluorescences were simultaneously measured. The obtained cytograms were analysed and the proportion of cells of the sample in each of the cell cycle compartments assessed [21].

Statistical analysis. Data are presented as the mean ± the standard error of three independent experiments. Means were compared using two-tail paired t-test. A difference was considered significant if a 0.05 value was reached.

Results

Cell survival. Survival rate was determined in both WiDr and MCF-7 cells pre-exposed for 72 hours to 5-FU and nucleoside analogues alone, or in combination. 5-FU alone was cytotoxic in both human tumor cell lines, although MCF-7 appeared to be more resistant than WiDr. The IC_{50} for 5-FU was 9 µM for WiDr and 130 µM for MCF-7, respectively. In WiDr cells the IC_{50}s for AZT, D4T, DDC and IdUrd were 535µM, 534µM, 720µM and 190µM, respectively. However, in MCF-7 cells IC_{50} for various nucleoside analogues could not be achieved even at concentrations up to 600µM.

The combined cytotoxicity of 5-FU with AZT against WiDr and MCF-7 cells is shown in Figure 1A and 2A, respectively. A 3-day exposure to AZT resulted in a significant increase in 5-FU cytotoxicity in both cell lines in a dose-dependent manner. Combinations of 100µM AZT with various concentrations of 5-FU produced a 1.6- to 2.4-fold increase in cytotoxicity of 5-FU in WiDr cells. The addition of 5µM and

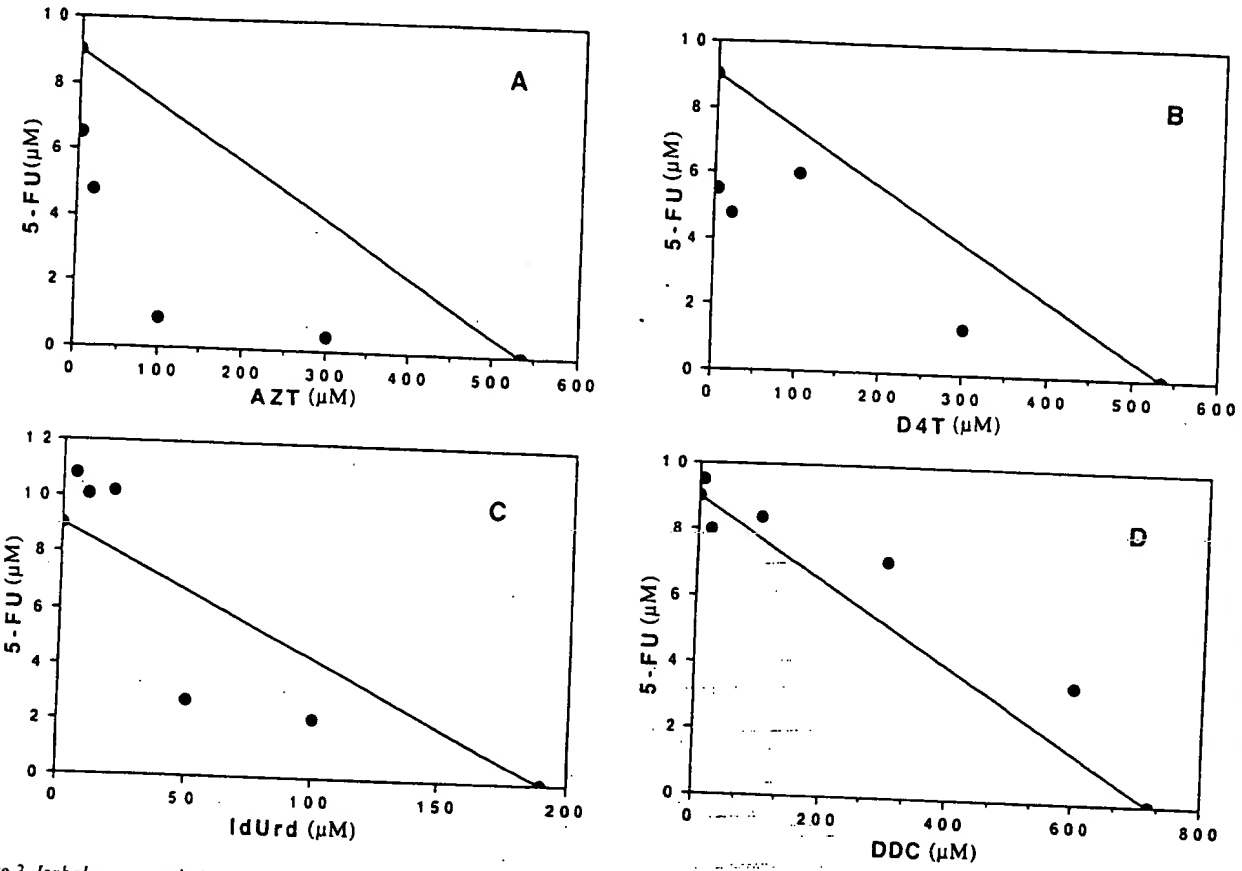

Figure 3. *Isobologram analysis of the cytotoxic effects of the combination of 5-FU and AZT (A), D4T (B), IdUrd (C) or DDC (D) in WiDr cells. Interaction of 5-FU and AZT or D4T is synergistic over the entire range of concentrations tested (5-300 μM), while the interaction between 5-FU and IdUrd is antagonistic over the lower concentrations of durgs tested (5-20 μM) and synergistic at higher concentrations (50 and 100 μM). DDC antagonized the 5-FU cytotoxicity over nearly the entire range of concentrations tested (10-600 μM).*

100μM AZT to various concentrations of 5-FU lowered the IC_{50} of 5-FU to 6.5 and 0.9 μM in WiDr cells, respectively. The isobologram analysis indicates that the combination of 5-FU and AZT exert a synergistic effect against WiDr (Figure 3A). Although the IC_{50} for nucleoside analogues cannot be achieved even at concentration up to 600 μM in MCF-7 cells, the IC_{40} isobologram assay show the same synergistic effect of AZT and 5-FU (Figure 4A).

In WiDr cells the IC_{50} of D4T after a 3-day continuous exposure was 534 μM. Figure 1B and 2B show the survival rate of 5-FU with or without D4T in WiDr and MCF-7. When WiDr cells were incubated with 5-FU and D4T, a supra-additive cytotoxic effect was observed (Figure 3B). D4T at concentrations of 5 and 300μM, used concurrently with 5-FU, lowered the IC_{50} for 5-FU to 5.5 and 1.4μM on WiDr cells, respectively. Compared to 5μM AZT, stronger synergism was obtained when 5μM D4T was added to the medium containing various concentrations of 5-FU (Figure 3A and 3B). On MCF7 cells, cytotoxicity assay revealed results similar to those with WiDr cells (Figure 4B).

IdUrd was more cytotoxic to WiDr and MCF-7 cells than AZT, DDC and D4T (Figure 1C and 2C). At a concentration of 10μM, 12.8±2.4% WiDr cells and 28.8±0.2% of MCF-7 cells were killed, respectively. The addition of 50μM IdUrd to 5-FU lowered the IC_{50} of 5-FU to 2.71μM in WiDr cells. The combination of 5-FU and IdUrd was synergistic over the entire range of concentrations of IdUrd in C113C of- MCF-7 cells, but was \only synergistic for the higher concentrations of IdUrd (50 and 100μM) against WiDr cells (Figures 3C and 4C). Moreover, at lower concentrations of IdUrd (5, 10 and 20mM), the interaction with IdUrd and 5-FU was antagonistic for WiDr cells (Figures 3C).

DDC was cytotoxic to WiDr cells with IC_{50} of 720μM, whereas on MCF-7 cells DDC produced no growth inhibition with concentrations up to 600μM (Figures 1D). When DDC was combined with 5-FU, DDC reduced the cytotoxic effect of 5-FU on WiDr cells for the entire range of concentrations of DDC (Figures 3D). In addition, the combination of DDC below toxicity level seemed to offer no potential effect on 5-FU (data not shown).

Cell cycle effect. Cells pre-treated with nucleoside analogues

Table I. *Percentage S-phase distribution in the cell cycle following exposure of cells to AZT, D4T and DDC.*

		24 h	48 h	72 h
WiDr	Control	36.2 ± 4.8	30.7 ± 1.7	30.0 ± 1.6
	AZT	36.5 ± 1.6	39.0 ± 1.0**	33.6 ± 1.9*
	D4T	36.3 ± 2.4	40.4 ± 0.6**	39.6 ± 1.3**
	DDC	37.0 ± 2.4	37.2 ± 1.0**	35.1 ± 1.5**
MCF-7	Control	37.9 ± 2.2	36.2 ± 1.9	28.0 ± 1.1
	AZT	40.8 ± 2.4*	34.9 ± 1.8	29.2 ± 0.6
	D4T	41.0 ± 3.2	36.3 ± 1.8	26.5 ± 0.8
	DDC	37.3 ± 2.6	35.1 ± 1.9	25.6 ± 1.4

Data represent with mean ± SE in three independent experiments in triplicate tests
* P<0.05 relative to control
** P<0.01 relative to control

were labelled with BrdUrd prior to fixation and processed as described. For the WiDr cell line, we observed an accumulation of cells in S-phase, mainly at 48 hours incubation with nucleoside analogues (Table I). No difference could be shown between early or late S (data not shown) . In contrast, no differences in cell cycle distributions were detected with MCF-7 and hence the modulatory effect of D4T and AZT on this latter cell line cannot be explained by cell synchronisation.

Discussion

In the present study, the cytotoxic interaction of 5-FU and various nucleoside analogues has been investigated *in vitro*. The combination of 5-FU with thymidine analogues such as AZT, D4T or IdUrd has a supra-additive cytotoxic effect on human colon and breast cancer cells. In contrast, DDC protects exponentially growing WiDr against the cytotoxic effects of 5-FU.

The major biochemical mechanism by which 5-FU produces cytotoxic and therapeutic effect is the inhibition of TS, a key enzyme in the *de novo* synthesis of thymidine triphosphate (dTTP). This depletion of dTTP can be partially circumvented by the salvage pathway and especially by the increase in the activity of thymidine kinase (TK) [22,23]. It has been shown that dTTP depletion by TS inhibition increases the incorporation of AZT triphosphate into DNA, leading to DNA chain termination [4,5,24]. In addition, the increased TK activity and nucleoside transporter expression as suggested by Dornowski and Pressacco may favor AZT incorporation into DNA [22,23]. On the other hand, Weber *et al* suggested that AZT is able to block the salvage pathway by competitively inhibiting TK. However, considering the Ki, AZT has a minor direct inhibitory effect on TK [5-8].

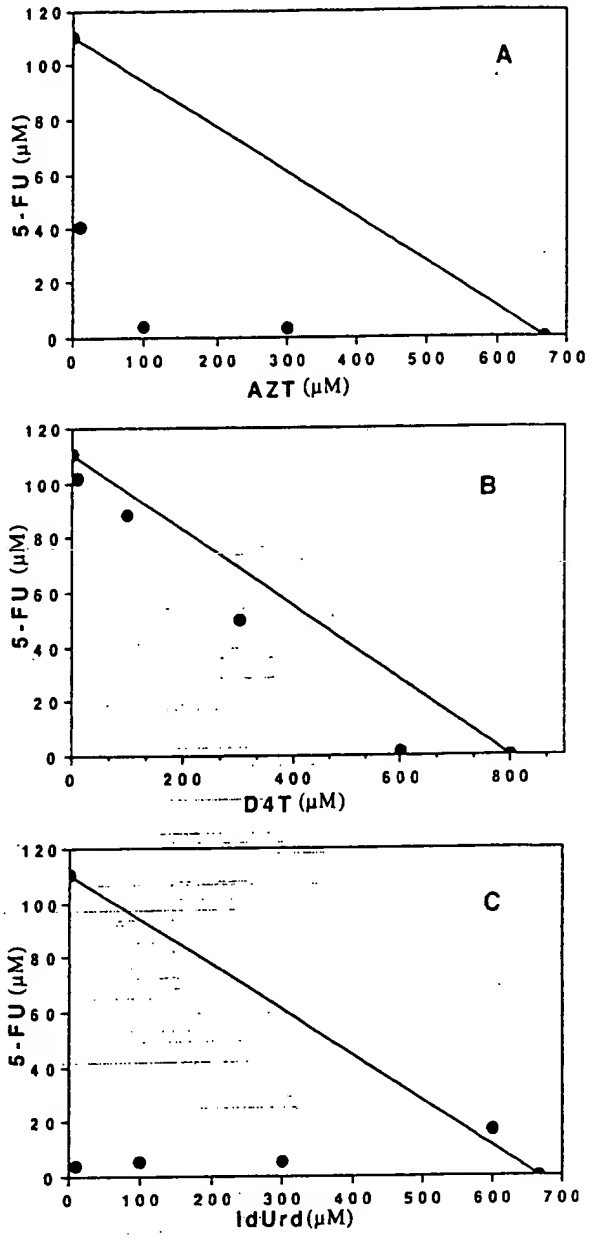

Figure 4. *Isobologram analysis of the cytotoxic effects of the combination of 5-FU and AZT (A), D4T (B) or IdUrd (C) in MCF-7 cells. Interaction of 5-FU and AZT, D4T or IdUrd is synergistic over nearly the entire range of concentrations tested (10-600 μM).*

AZT and D4T are phosphorylated by the same kinases that activate thymidine [16]. Once phosphorylated to the triphosphate derivatives, they are incorporated into DNA, leading to chain termination [4]. As is the case with AZT, there is increased incorporation of D4T into DNA as a result of a 5-

FU-mediated reduction in the intracellular dTTP pool [23]. One may observe that the similarity between AZT and D4T metabolism is one way of explaining comparable effects on 5-FU's toxicity on human breast and colon cancer cells. However, the use of D4T as a modulator offers a potential advantage over AZT. First, in contrast to AZT, D4T does not accumulate in its monophosphate form because it lacks an inhibitory effect on dTMP-kinase (less toxicity on bone marrow) [16,25]. Second, the lack of accumulation of D4T-MP allows better incorporation into DNA, which is required for optimal interaction with 5-FU. One could therefore expect that D4T on this theoretical basis will have a better therapeutic index than AZT, but that remains to be investigated *in vivo*.

IdUrd is known to be active as a radiosensitizer and cytotoxic drug, and its activity depends on the amount of thymidine replacement [26-29]. On the other hand, IdUrd monophosphate is a weak inhibitor of TS [29]. Moreover, some investigators have demonstrated an increased phosphorylation and incorporation of IdUrd into DNA after TS inhibition mediated by 5-FU, fluorodeoxyuridine or Tomudex. A supra-additive effect has been observed both *in vitro* and *in vivo* [9,28]. Our data are therefore consistent with other investigators.

DDC is a cytosine derivative. The metabolic pathway is different compared to thymidine analogues [25]. It has been proposed as an alternative to AZT in non-responding AIDS patients [17]. DDC, even at a high doses, has minimal or no toxic effect on WiDr and MCF-7 cells. Moreover, it antagonizes the effect of 5-FU.

The combined effect of 5-FU and AZT, D4T, IdUrd or DDC *in vitro* is dependent on the type of the nucleoside, the cell-line tested and the concentration used. This suggests that by affecting one or multiple enzymes or natural substrates involved in either 5FU metabolism or in purine or pyrimidine biosynthesis, a different modulating effect can be observed. On the basis of these findings, AZT, D4T and IdUrd are more likely to be subjected to more intensive *in vitro* and *in vivo* research as far as modulation of 5-FU toxicity is concerned. Taken together these results suggest that some nucleoside analogues can be exploited as potential modulators of 5-FU toxicity. As recently reviewed by Jackman and Calvert, many folate-based TS inhibitors are developed because of the growing evidence of the central role of TS in neoplastic growth [30]. The efficacy of these TS-inhibitors can be reduced by the salvage pathway and therefore development of combinations of 5FU or new TS-inhibitors and nucleoside analogues, aimed at being incorporated into DNA in order to lead to chain termination, are of practical and clinical interest.

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References

1 Wadler S, Wersto R, Weinberg V, Thompson D, Schwartz Interaction of fluorouracil and interferon in human colon cancer lines: cytotoxic and cytokinetic effects. Cancer Res *50*: 5735-5 1990.

2 Hainsworth JD: The use of mitoxantrone, 5-fluorouracil and l dose leucovorin in the treatment of advanced breast cancer. Oncol *4*(Suppl. 2): S37-S40, 1993.

3 Naguib ENM, Hao SN, Kouni MH: Potentiation of 5-fluorou efficacy by the dihydrouracil dehydrogenase inhibitor, 5-benzyl benzyluracil. Cancer Res *54*: 5166-5170, 1994.

4 Brunetti I, Falcone A, Calabresi P, Goulette FA, Darnowski JW fluorouracil enhances azidothymidine cytotoxicity: *In vitro*, *in* and biochemical studies. Cancer Res *50*: 4026-4031, 1990.

5 Tosi P, Calabresi P, Goulette FA, Renaud CA, Darnowski Azidothymidine-induced cytotoxicity and incorporation into DN the human colon tumor cell line HCT-8 is enhanced by methotre *in vitro* and *in vivo*. Cancer Res *52*: 4069-4073, 1992.

6 Weber G, Ichikawa S, Nagai M, Natsumeda Y: Azidothymi inhibition of thymidine kinase and synergistic cytotoxicity methotrexate and 5-fluorouracil in rat hepatoma and human c cancer cells. Cancer Commun *2*: 129-133, 1990.

7 Weber G, Nagai M, Prajda N, Nakamura H, Szekeres T, Ola AZT: a biochemical response modifier of methotrexate an fluorouracil cytotoxicity in human ovarian and pancreatic carcin cells. Cancer Commun *3*: 127-132, 1991.

8 Zhen YS, Taniki T, Weber G: Azidothymidine and dipyridamo biochemical response modifiers: synergism with methotrexate a fluorouracil in human colon and pancreatic carcinoma cells. C Res *4*: 73-78, 1992.

9 Benson III AB, Trump DL, Cummings KB, Fischer PH: Modul of 5-iodo-2'-deoxyuridine metabolism and cytotoxicity in hu bladder cancer cells by fluoropyrimidines. Biochem Pharmaco 3925-3931, 1985.

10 Scanlon KJ, Kashani-Sabet M, Sowers LC: Overexpression of l replication and repair enzymes in cisplatin-resistant human c carcinoma HCT-8cells and circumevention by azidothymi Cancer commun *1*: 269-275, 1989.

11 Szekeres T, Findenig G, Fritzer-Szekeres M, Mader R, Gohl R, is capable of modulating 5-FU resistance in human colon tumor Proc Am Assoc Cancer Res *37*: 293, 1996.

12 Posner MR, Darnowski JW, Calabresi P, Brunetti I, Corvese D, G, Cummings FJ, Clark J, Browne MJ, Beitz J, Weitberg AB: zidovudine, continuous-infusion fluorouracil, and oral leucov calcium: a phase I study. J Natl Cancer Inst *82*: 1710-1714, 1990.

13 Posner MR, Darnowski JW, Weitberg AB, Dudley MN, Corves Cummings FJ, Clark J, Murray C, Clendennin N, Bigley J, Cala P: High-dose intravenous zidovudine with 5-fluorouracil and l vorin: a phase I trial. Cancer *70*: 2929-2934, 1992.

14 Falcone A, Danesi R, Dargenio F, Pfanner E, Brunetti I, Tacca Nethersell ABM, Conte PF: Intravenous azidothymidine fluorouracil and leucovorin: a phase I-II study in previously untr metastatic colorectal cancer patients. J Clin Oncol *14*: 729-736, 1

15 Chang AE, Collins JM, Speth PAJ, Smith R, Rowland JB, Cam L, Begley MG, Glatstein E, Kinsella TJ: Phase I study of intraar iododeoxyuridine in patients with colorectal liver metastases. J Oncol *7*: 662-668, 1989.

16 Balzarini J, Herdewijn P, De Clercq E: Differential patter intracellular metabolism of 2',3'-didehydro-2',3'dideoxythym

(D4T) and 3'-azido-2',3'-dideoxythymidine (AZT), two potent anti-HIV compounds. J Biol Chem *264:* 6127-6133, 1989.

17 Bozzette SA, Kanouse DE, Berry S, Duan N: Health status and function with zidovudine or zalcitabine as initial therapy for AIDS: a randomized controlled trial. Roche 3300/ACTG 114 Study Group. JAMA *273:* 295-301, 1995.

18 Denizot F, Lang R: Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods *89:* 271-277, 1986.

19 Chou TC, Talalay P: Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs and enzyme inhibitors. Adv Enzyme Regul *22:* 27-55, 1984.

20 Begg AC, NcNally NJ, Shrieve DC, Karcher H: A method to measure the duration of the DNA synthesis and the potential doubling time from a single sample. Cytometry *6:* 620-626, 1985.

21 McGinn CJ, Miller EM, Lindstrom MJ, Kunugi KA, Johnston PG, Kinsella TJ: The role of cell cyle redistribution in radiosensitization: implications regarding the mechanism of fluorodeoxyuridine radiosensitization. Int J Radiat Oncol Biol Phys *30:* 851-859, 1994.

22 Darnowski JW, Brunetti I, Posner M, Weitberg A, Falcone A, Goulette F, Corvese M, Calabresi P: Fluorouracil plus azidothymidine cytotoxicity *in vitro*: relationship to cellular thymidine kinase activity. Proc Am Assoc Cancer Res *31:* 110, 1990.

23 Pressacco J, Mitrovski B, Erlichman C, Hedley DW: Effects of thymidylate synthase inhibition on thymidine kinase activity and nucleoside transporter expression. Cancer Res *55:* 1505-1508, 1995.

24 Pressacco J, Erlichman C: Combination studies with 3'-azido3'-deoxythymidine (AZT) plus ICI D1694: cytotoxic and biochemical effects. Biochem Pharmacol *46:* 1989-1997, 1993.

25 Balzarini J: The metabolism and mechanism of antiretroviral action of purine and pyrimidine derivatives. Pharm World Sci *16:* 113-126, 1994.

26 Lawrence TS, Davis MA, Maybaum J, Stetson PL, Ensminger WD: Modulation of iododeoxyuridine-mediated radiosensitization by 5-fluorouracil in human colon cancer cells. Int J Radiat Oncol Biol Phys *22:* 499-503, 1992.

27 Kuo ML, Kunugi KA, Lindstrom MJ, Kinsella TJ: The interaction of hydroxyurea and iododeoxyuridine on the radiosensitivity of human bladder cancer cells. Cancer Res *55:* 2800-2805, 1995.

28 Pressacco J, Hedley DW, Erlichman C: ICI D1694 and idoxuridine: a synergistic antitumor combination. Cancer Res *54:* 3772-3778, 1994.

29 Greene RF, Collins JM: Effects of leucovorin on idoxyuridine cytotoxicity and DNA incorporation. Cancer Res *50:* 6652-6656, 1990.

30 Jackman AL, Calvert AH: Folate-based thymidylate synthase inhibitors as anticancer drugs. Ann Oncol *6:* 871-881, 1995.

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Metabolism and DNA Interaction of 2′,3′-Didehydro-2′,3′-dideoxythymidine in Human Bone Marrow Cells

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SUMMARY

2′,3′-Didehydro-2′,3′-dideoxythymidine (D4T) is a potent inhibitor of human immunodeficiency virus (HIV), with low hematological toxicity. In the present study, the cellular pharmacology of D4T was investigated in human bone marrow cells (BMC), in an attempt to understand the mechanism of the observed low bone marrow toxicity. After exposure of human BMC to 10 μM [^{3}H] D4T for 24 hr, D4T-5′-triphosphate (D4T-TP) was the predominant metabolite, reaching a concentration of 0.3 pmol/10^6 cells. The D4T-5′-monophosphate levels were slightly lower, whereas the D4T-5′-diphosphate levels were about 6-fold lower than those of D4T-TP at 24 hr. Nucleic acids of human BMC exposed to 10 μM [^{3}H]D4T for 24 hr were purified and analyzed by cesium sulfate density gradient centrifugation. No radioactivity was detected in the RNA region, whereas a limited amount was associated with the DNA region. The amount of label incorporated into DNA correlated with the extracellular D4T concentration and the length of incubation time. Enzymatic hydrolysis of radiolabeled DNA and subsequent analysis by high performance liquid chromatography demonstrated incorporation of both D4T and thymidine (dThd) into DNA. Degradation of D4T to thymine and subsequent formation of labeled dThd was also detected in human BMC. Pulse (24 hr)-chase (48 hr) experiments with 10 μM [^{3}H]D4T demonstrated that the amount of radiolabel from D4T in DNA decreased over time during the chase. Under similar conditions, [^{3}H]3′-azido-3′-deoxythymidine (AZT) incorporated into DNA of human BMC did not decrease during the chase. Although D4T-TP standard was demonstrated to be unstable at 37° and neutral pH, D4T was much more stable in solution and incorporated into newly synthesized DNA isolated from human BMC, suggesting that enzymatic excision may be the mechanism for D4T removal from DNA. In summary, although higher concentrations of D4T-TP, compared with AZT-5′-triphosphate, are observed in human BMC, after exposure of cells to similar extracellular concentrations of parent drug, steady state levels of D4T incorporated into DNA are 10–50-fold lower, compared with AZT. Competition with dTTP formed by D4T metabolism and excision of D4T from DNA may be responsible, in part, for these effects. This study further demonstrates that incorporation of 2′,3′-dideoxynucleosides into nuclear DNA of human BMC may be related to the ability of these anti-HIV agents to induce hematological side effects.

Development of anti-HIV agents with a high degree of selectivity represents a major approach to preventing and/or treating AIDS. AZT is currently the only clinically approved drug for the treatment of AIDS, but its use in patients has been hampered by its severe hematological toxicity (1–3). Studies over the past years have demonstrated the importance of the intracellular metabolism of AZT and how this metabolism plays a major role in both the antiviral and the cytotoxic effects of AZT in host cells (4–10).

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D4T is a novel 2′,3′-dideoxynucleoside that has been shown in several *in vitro* systems to inhibit HIV replication, at concentrations below 0.01 μM, with limited host cell toxicity (11, 12). In particular, we demonstrated that D4T had IC_{50} values for human CFU-GM and human erythroid burst-forming units 100- and 10-fold, respectively, higher than those of AZT (13); thus, D4T was predicted to show less bone marrow suppression.

That prediction proved accurate in animal studies (14) and phase I clinical trials (15–17). In patients, following administration of D4T at doses up to 4 mg/kg/day, preliminary indications of efficacy with reduced p24 antigenemia were observed without substantial bone marrow toxicity (15–17). However,

ABBREVIATIONS: HIV, human immunodeficiency virus; D4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; D4T-MP, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-monophosphate; D4T-DP, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-diphosphate; D4T-TP, 2′,3′-didehydro-2′,3′-dideoxythymidine triphosphate; AZT, 3′-azido-3′-deoxythymidine; AZT-MP, 3′-azido-3′-deoxythymidine 5′-monophosphate; AZT-TP, 3′-azido-3′-deoxythymidine triphosphate; dThd, thymidine; BMC, bone marrow cells; AIDS, acquired immune deficiency syndrome; HPLC, high performance liquid chromatography; CFU-GM, colony-forming unit granulocyte-macrophage; TCA, tricholoracetic acid.

unpredicted peripheral neuropathy, already reported with 2′,3′-dideoxycytidine and 2′,3′-dideoxyinosine, was the major limiting toxicity (15–17). Research on the mechanism(s) by which these dideoxynucleosides, including D4T, induce this neurotoxicity suggests that their effects on mitochondrial DNA synthesis may play a role in the observed neuropathies (18, 19). The absence of D4T-induced myelosuppression is particularly interesting, because other dThd analogs, including 3′-fluoro-3′-deoxythymidine, AZT, and its recently identified metabolite 3′-amino-3′-deoxythymidine, have been demonstrated to be highly toxic for human BMC (20, 21). Previous studies have suggested that differences in metabolism of D4T and AZT may be responsible for the decreased host toxicity (8, 22). AZT, unlike D4T, accumulates as its 5′-monophosphate derivative within cells, and these high intracellular AZT-MP levels may lead to inhibition of dTMP kinase. This, in turn, results in reduction of dTTP pools (8, 23), a mechanism that has not been observed with D4T (8, 24). However, our recent studies using human BMC and a pharmacologically relevant concentration of 10 μM AZT demonstrated that imbalance of deoxyribonucleotide pools was not a critical factor in AZT inhibition of DNA synthesis (4). These data were also confirmed by several other groups using different cell lines and experimental procedures (5, 7, 10, 25). In contrast, although AZT is a poor substrate for human DNA polymerase α and δ (25–27), substantial amounts of that drug are incorporated into nuclear DNA (4, 25, 28), and this biochemical event has been demonstrated by us to correlate, to some degree, with inhibition by AZT of CFU-GM colony formation (4). The decreased toxicity of D4T in that system may, thus, result from a low steady state level of D4T incorporation into host cellular DNA, possibly subsequent to poor phosphorylation of D4T in human BMC and/or a different interaction of D4T with DNA, compared with AZT.

Therefore, in the present study we evaluated the intracellular metabolism and incorporation into nucleic acids of D4T in human BMC. The pattern of D4T phosphorylation to its 5′-phosphate derivatives was similar to that observed in other human primary cells (29) or established cell lines (8, 22, 30). Of note, degradation of D4T to thymine and subsequent formation of dThd was demonstrated within cells, consistent with the detection of labeled dThd in DNA of cells that had been exposed to labeled D4T. Steady state levels of D4T incorporated into DNA were 10–50-fold lower, compared with AZT, after exposure of cells to similar concentrations. Removal of D4T from DNA was detected under conditions in which no excision of AZT was detected.

Materials and Methods

Chemicals. [*methyl*-^{3}H]D4T (20 Ci/mmol), [*methyl*-^{3}H]AZT (11 Ci/mmol), and [*methyl*-^{3}H]dThd (65 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). The purity of all radiolabeled compounds used was >99%, as ascertained by HPLC techniques described below. Nonlabeled standard AZT was a generous gift of Dr. Raymond Tennant (Veterans Administration Medical Center and Emory University, Atlanta, GA). D4T and its nucleotides were chemically synthesized as previously described (12). Nucleosides, RNase A and T_1, proteinase K, alkaline phosphatase, and snake venom and spleen phosphodiesterase were obtained from Sigma Chemical Co. (St. Louis, MO). Micrococcal nuclease was purchased from Worthington Biochemical (Freehold, NJ). All other chemicals and reagents were of the highest analytical grade available.

Cells and extraction of intracellular nucleotides. Human l were obtained by procedures described in detail by us previously Cell viability, as determined by trypan blue dye exclusion, was 95 greater during experiments. Cells (2×10^6 cells/ml) were suspend McCoy's 5A nutrient medium supplemented with 15% dialyzed h inactivated fetal bovine serum. After addition of various concentrat of D4T, cells were maintained at 37° under an atmosphere of 5% (for specified time periods. Cells were then pelleted at 1200 rpm fo min, in a Beckman GPR centrifuge, and were washed three times 5 ml of cold phosphate-buffered saline. Nucleotides present in the pellet were extracted by incubation overnight at −20° with 1 ml of methanol and were then extracted with 200 μl of 60% methanol fo min in an ice bath. Combined extracts were dried under a ge nitrogen stream at room temperature, and the samples were store −20° until analysis.

HPLC methods. Separation of nucleotides was performed o Hewlett-Packard 1090 HPLC system equipped with automatic injec filter spectrophotometric detector, and chromatographic termi (Hewlett Packard 3393A). Analysis of D4T and its 5′-phosphoryla derivatives, and cell extracts containing them, was performed usin Partisil 10 SAX column (Whatman, Inc., Clifton, NJ) as station phase. Elution was carried out at 1 ml/min with 15 mM KH_2PO_4 (3.5) and a 45-min linear gradient of 1 M KH_2PO_4 (pH 3.5) from 0 100%, starting 10 min after the time of injection. Under the conditic defined above, the retention times of the unlabeled markers D4T, D4 MP, and D4T-TP were 7, 14, and 54 min, respectively. The assignme of D4T-DP at 33 min was assumed from its position between the mor and triphosphate derivatives and its dephosphorylation to D4T enzymatic hydrolysis with alkaline phosphatase, as previously d scribed (22). For sample analysis, dried residues were dissolved in 2 μl of distilled water, and an aliquot (180 μl) was injected. After fra tionation (1 ml) of the eluate and addition of scintillation fluor (5 m radioactivity was measured by using a Beckman LS 5000TA scintill tion counter equipped with an automatic quench correction progra Reverse phase chromatography was used to separate D4T or AZT fro thymine and dThd. Extracts were examined using a Hypersil 5-μ column (Jones Chromatography, Littleton, CO) as stationary phas Elution was carried out at 1 ml/min with 25 mM ammonium phospha (pH 7.2) and a 35-min linear gradient of 60% methanol from 0 to 30% starting at the time of injection. The retention times of authenti standards of thymine, dThd, D4T, and AZT were 11, 21, 27, and 3 min, respectively.

Incorporation of D4T into nucleic acids of human BMC. T examine D4T incorporation into DNA, cells (2×10^6 cells/ml) wer incubated at 37° for 24 hr after addition of various concentrations o [^{3}H]D4T, from 1 to 25 μM, with a final specific activity of approximatel 800 mCi/mmol. DNA was extracted by phenol/chloroform and protein ase K and RNase A and T_1 digestion procedures, as previously describe (4). The amount of DNA in each sample was determined by a fluorom etry technique that includes binding of bisbenzimidazole to DNA (31). Samples (100 μl) were then spotted on Whatman 3MM filter paper and filters were washed twice with ice-cold 5% TCA, followed by 100% methanol. Filters were dried at room temperature, and radioactivity was determined in a Beckman LS 5000 TA scintillation counter.

Cesium sulfate density gradient centrifugation. Cellular nucleic acids were extracted and analyzed by cesium sulfate gradient centrifugation, as described previously (4).

Enzymatic digestion of ^{3}H-labeled DNA. After cesium sulfate density gradient centrifugation, fractions with a density of 1.41–1.43 g/ml were pooled and dialyzed overnight against 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, after which DNA was precipitated in 0.3 M sodium acetate and ice-cold absolute ethanol. DNA was then dissolved in 20 μl of Tris·HCl (pH 7.4), 1 mM EDTA, and heat denatured by boiling at 100° for 5 min. The DNA was hydrolyzed at 37° for 30 min with 50 μl of micrococcal nuclease (12 units/ml), in 10 mM Tris·HCl (pH 8.8), 2 mM $CaCl_2$. After addition of a mixture containing

degradation to nucleosides was carried out at 37° for 24 hr, with snake venom 5′-phosphodiesterase (2 units), spleen 3′-phosphodiesterase (2 units), and alkaline phosphatase (0.3 units). The digested material was then analyzed by the reverse phase HPLC method described above.

Hydrolysis of DNA to nucleoside monophosphates was carried out at 37° by sequential action of micrococcal nuclease (12 units/ml) and spleen 3′-phosphodiesterase (2 units). The fraction containing the radioactive nucleoside monophosphates was analyzed by anion exchange HPLC technique, except that a different elution mode was used. Elution was performed isocratically at 0.5 ml/min with 15 mM K_2HPO_4 (pH 3.5) for 20 min, followed by a 45-min linear gradient of 1 M K_2HPO_4 (pH 3.5) from 0 to 100%. The retention times for dThd 5′-monophosphate, dThd 3′-monophosphate, and D4T-MP were 15, 18, and 21 min, respectively.

Pulse-chase labeling of DNA with ^{3}H-nucleosides. Human BMC were exposed to either 10 μM [^{3}H]D4T (360 mCi/mmol), 10 μM [^{3}H]AZT (360 mCi/mmol), or 10 μM [^{3}H]dThd (360 mCi/mmol), for 24 hr at 37°. After the 24-hr pulse, the radioactive medium was removed and the cells were washed with a fresh phosphate-buffered saline solution at 0°. The cells were then chased for 0, 6, 24, and 48 hr at 37°, in McCoy's 5A nutrient medium containing 10 μM (nonlabeled) levels of the corresponding nucleoside or nucleoside analog being investigated. The chase was terminated by pelleting of cells and placement in an ice-cold water bath. Radioactivity was then analyzed in total DNA, following procedures described above.

Degradation of D4T by cell extracts. Catabolic conversion of D4T to thymine was investigated in human BMC using a modified radioisotopic assay (32). Human BMC (80×10^6 cells/ml) were lysed by two cycles of 30-sec sonic oscillation at 0°, in 0.5 ml of 2 mM potassium phosphate (pH 7.4). The reaction consisted of either 2 μM [^{3}H]D4T (450 mCi/mmol), 2 μM [^{3}H]AZT (450 mCi/mmol), or 2 μM [^{3}H]dThd (450 mCi/mmol), in 100 mM Tris·HCl (pH 6.0), 12.5 mM potassium phosphate (pH 6.0), with cell extract (60 μl), in a final volume of 150 μl. Boiled cell extracts were used as negative control. The reaction was performed at 37° for specified time periods. Aliquots (25 μl) were extracted overnight at −20° with cold 60° methanol. Samples were dried and injected into HPLC using the reverse phase method described above, which resolves thymine, dThd, D4T, and AZT.

Stability of labeled D4T in cellular DNA. Cells (2×10^6 cells/ml) were incubated for 24 hr with 10 μM [^{3}H]D4T, at a final specific activity of 450 mCi/mmol, and DNA was purified as described above. The DNA extracted from 20×10^6 cells was dissolved in 0.5 ml of a buffer containing Tris·HCl (pH 7.4) and 1 mM EDTA, to which was added 1.5 ml of McCoy's 5A nutrient medium. Mixtures were incubated at 37° for times between 0 and 48 hr. Aliquots (0.5 ml) were spotted on Whatman 3MM discs, and radioactivity was determined after treatment with TCA and methanol as described above. Stability of D4T-TP was also investigated in McCoy's 5A nutrient medium, at similar time intervals between 0 and 48 hr. D4T-TP concentrations were determined using the anion exchange HPLC technique described above.

Results

Metabolism of D4T in human BMC. After exposure of human BMC to 10 μM [^{3}H]D4T, D4T was rapidly phosphorylated within cells to its 5′-mono-, 5′-di-, and 5′-triphosphate derivatives, as revealed by anion exchange chromatography (Fig. 1A). The intracellular concentrations of D4T and its 5′-phosphate metabolites after 2-, 6-, 24-, and 48-hr incubation periods are illustrated in Table 1. A continuous increase in D4T-MP, D4T-DP, and D4T-TP levels was observed between 2 and 24 hr, and an equilibrium was maintained for the remaining 24 hr of the experiment. We previously demonstrated that the percentage of BMC in S phase did not change over the first 24 hr (4), thus demonstrating that the increase in D4T phosphorylated metabolites is not the result of an increased activity of dThd kinase, the enzyme proposed to be responsible in D4T activation (22, 24). By 24 hr, D4T-TP was the predominant intracellular metabolite and reached a mean concentration of 0.3 pmol/10^6 cells. The earlier eluting fraction (Fig. 1A)

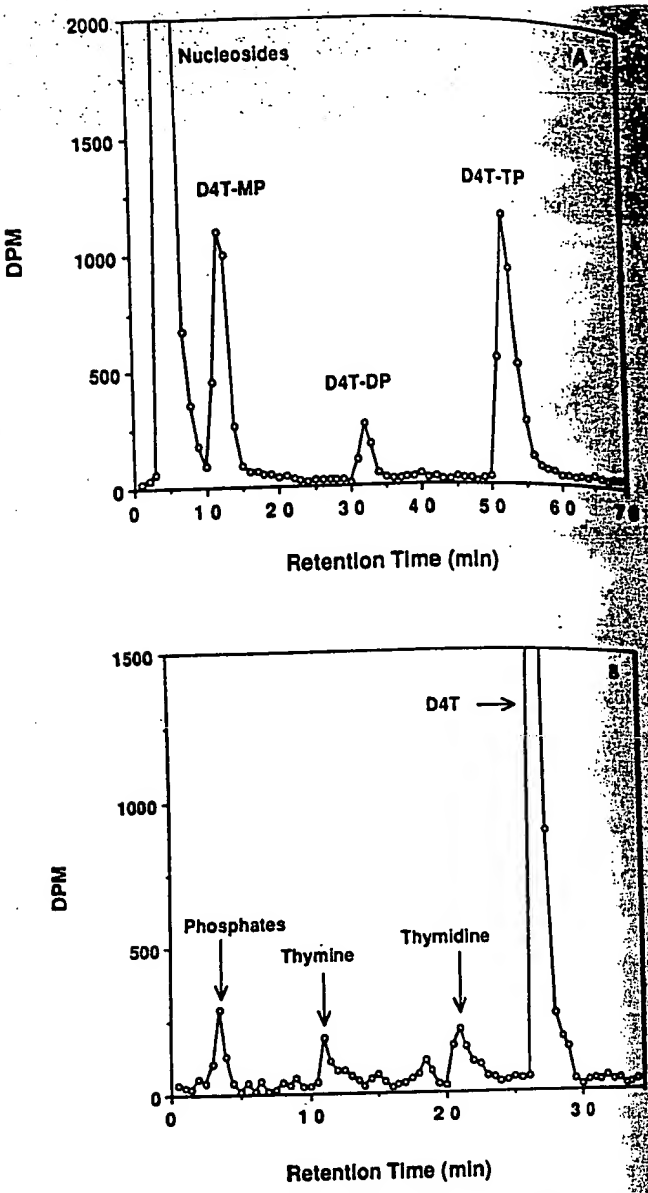


Fig. 1. Radiochromatogram of intracellular ^{3}H after exposure of human BMC for 24 hr to 10 μM [^{3}H]D4T and analysis by anion exchange HPLC (A) or analysis by reverse phase HPLC (B). Elution positions of authentic standards were monitored at 254 nm.

TABLE 1

Metabolism of 10 μM [^{3}H]D4T in human BMC

Values are mean ± standard deviation of at least three experiments, with cells from different donors.

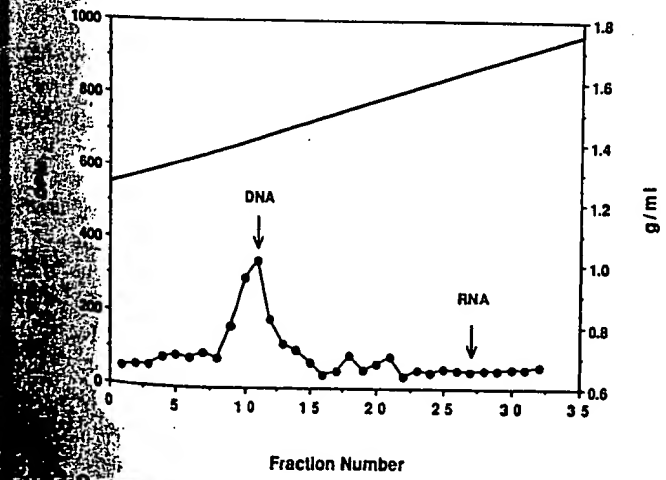
Time of exposure	Metabolite		
	D4T-MP	D4T-DP	D4T-TP
hr	*pmol/10^6 cells*		
2	0.129 ± 0.092	0.011 ± 0.007	0.043 ± 0.033
6	0.128 ± 0.047	0.019 ± 0.011	0.084 ± 0.062
24	0.267 ± 0.055	0.049 ± 0.042	0.300 ± 0.163
48	0.189 ± 0.087	0.052 ± 0.035	0.260 ± 0.095

coeluted with an authentic D4T standard and represented an average of 80–85% of the total intracellular radioactivity at every time point of sampling. Further analysis by reverse phase chromatography revealed that approximately 14% of that fraction actually represented thymine and dThd and only 80% was D4T (Fig. 1B). Because D4T used in these experiments was >98% pure, these data suggest that D4T may be cleaved within cells to release labeled thymine, with subsequent conversion to labeled dThd.

The extent of degradation of D4T was assessed by incubating 2 μM [^{3}H]D4T with human BMC extracts, as described in Materials and Methods. Parallel studies were performed with similar concentrations of [^{3}H]AZT and [^{3}H]dThd. After 48 hr of incubation and analysis by reverse phase HPLC, D4T represented only 69% of the total radioactivity, whereas thymine and dThd accounted for 25.5 and 3.5%, respectively. No substantial degradation of D4T was detected in control boiled cell extracts. Under similar conditions, dThd was completely converted to thymine, whereas AZT was very stable, with >95% of radioactivity being still associated with the parent drug. These data confirm that dThd phosphorylase activity is present in human BMC (33, 34) and indicate that D4T is probably a substrate for that enzyme. When purified dThd phosphorylase derived from *Escherichia coli* was used, D4T was also found to be a substrate for that enzyme.[1] In contrast, AZT was not degraded by bacterial dThd phosphorylase,[1] as suggested by cellular studies described above.

Analysis of [^{3}H]D4T incorporation into nucleic acids. In order to evaluate the interaction of D4T with host cell nucleic acids, human BMC were exposed for 24 hr to various concentrations of [^{3}H]D4T, from 1 to 25 μM, and extracted nucleic acids were partitioned by cesium sulfate density gradient centrifugation, as described in Materials and Methods. No radioactivity was detected in the RNA region, whereas a substantial amount of tritium label was associated with the DNA region (density, 1.42 g/ml) (Fig. 2). To determine quantitatively the amount of ^{3}H incorporated into DNA, as a function of extracellular D4T concentration and time of exposure, radioactivity was measured in purified DNA using a 'disk filter' assay, as previously described (4). As illustrated in Fig. 3, the amount of tritium incorporated into DNA correlated with the extracellular concentrations of [^{3}H]D4T used in the experiment (from 1 to 25 μM), and the amount of tritium associated with cellular DNA also increased from 0 to 24 hr, when a concentration of 10 μM [^{3}H]D4T was used.

Identification of ^{3}H detected in cellular DNA. Identification of the radioactivity detected in DNA was performed by enzymatically hydrolyzing the DNA to nucleosides or nucleoside monophosphates, with subsequent HPLC analysis. When the DNA was digested with micrococcal nuclease, snake venom and spleen phosphodiesterase, and alkaline phosphatase, two radioactive peaks, which coeluted with dThd and D4T standards, were detected by reverse phase HPLC analysis (Fig. 4). The ratio of D4T to dThd was approximately 1 to 3. To confirm the identification of D4T and dThd as the sole radioactive entities detected in DNA, another digestion strategy was used.



Cesium sulfate density gradient centrifugation of [^{3}H]D4T-radio- nucleic acids. After centrifugation of purified nucleic acids, frac- 200 μl were collected from top to bottom and 100-μl samples otted on Whatman 3-mm filter paper. Filters were washed and d radioactivity was determined.

Schinazi and J. P. Sommadossi, unpublished data.

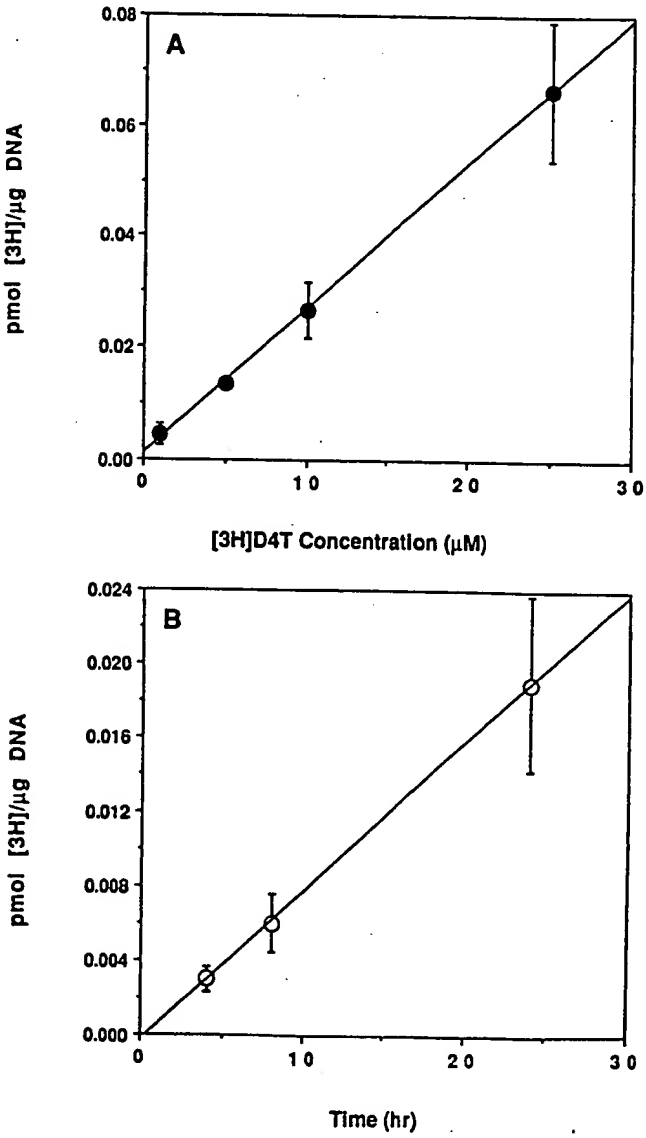


Fig. 3. Incorporation of radioactivity into DNA after incubation of cells for 24 hr with increasing concentrations of [^{3}H]D4T (A) and after incubation of cells with 10 μM [^{3}H]D4T for varying time periods (B) Values are the mean ± standard error of three experiments, with cells from different donors.

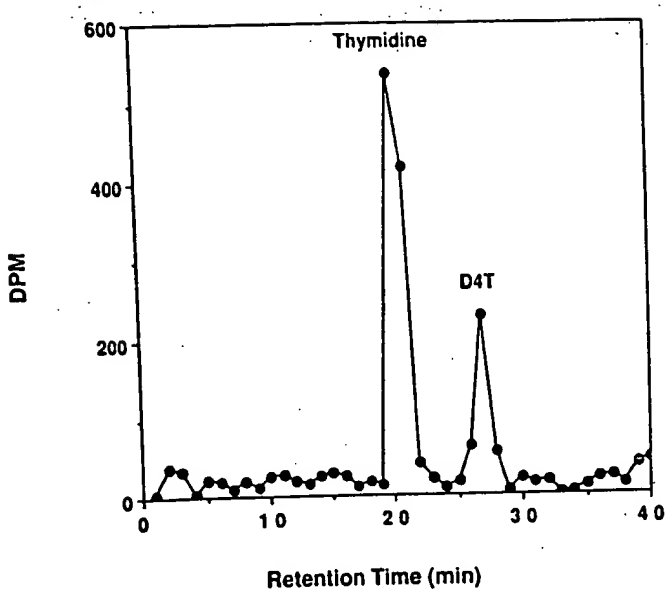


Fig. 4. Reverse phase HPLC analysis of ^{3}H-labeled digested DNA isolated from cells exposed to 10 μM [^{3}H]D4T for 24 hr. DNA digestion was performed by the sequential addition of micrococcal nuclease, snake venom and spleen phosphodiesterase, and alkaline phosphatase.

Briefly, after micrococcal nuclease digestion, only spleen 3′-phosphodiesterase was added to the reaction solution. This enzyme cleaves 5′-ester bonds and leaves the 3′-nucleoside monophosphates intact. Thus, under these conditions, nucleotides that are incorporated at the terminus of the DNA chain and have no 3′-hydroxyl group will be converted to their corresponding nucleosides rather than their 3′-monophosphates. Fig. 5 shows the radiochromatogram of the DNA digest after exposure of cells, for 24 hr, to either 10 μM [^{3}H]dThd or 10 μM [^{3}H]D4T. The dThd 3′-monophosphate represented the major radioactive component of the newly synthesized DNA after exposure to [^{3}H]dThd, consistent with the natural pyrimidine being localized in the intranucleotide linkage. In contrast, digestion of DNA isolated from cells treated with [^{3}H]D4T led to the detection of a portion of radioactivity eluting as a deoxyribonucleoside derivative and a major fraction corresponding to the dThd 3′-monophosphate region. These data confirm that labeled D4T and dThd are both present in newly synthesized DNA after exposure of cells to [^{3}H]D4T and demonstrate, as expected, that D4T accumulates at the chain termini of host cell DNA. Of note, the ratio of D4T to dThd was similar to that described above when different DNA hydrolysis and HPLC analysis were performed.

Removal of D4T from newly synthesized DNA in human BMC. In order to gain insight into potential mechanisms responsible for the rather small amount of D4T detected in cellular DNA of human BMC, compared with AZT (4), we investigated whether D4T could be removed from DNA of human BMC. Cells were incubated for 24 hr with either 10 μM [^{3}H]D4T or [^{3}H]AZT, and the label was then chased as described in Materials and Methods. Control experiments were also performed by incubating cells with [^{3}H]dThd. Fig. 6 illustrates the tritium remaining in DNA over the 48-hr chase in the presence of the corresponding nonlabeled compound. In control as well as in AZT experiments, no decrease of radioactivity in DNA was observed over the 48-hr chase period. In contrast, over this same interval, approximately 30–40% of the tritium in total DNA from D4T-treated cells was removed.

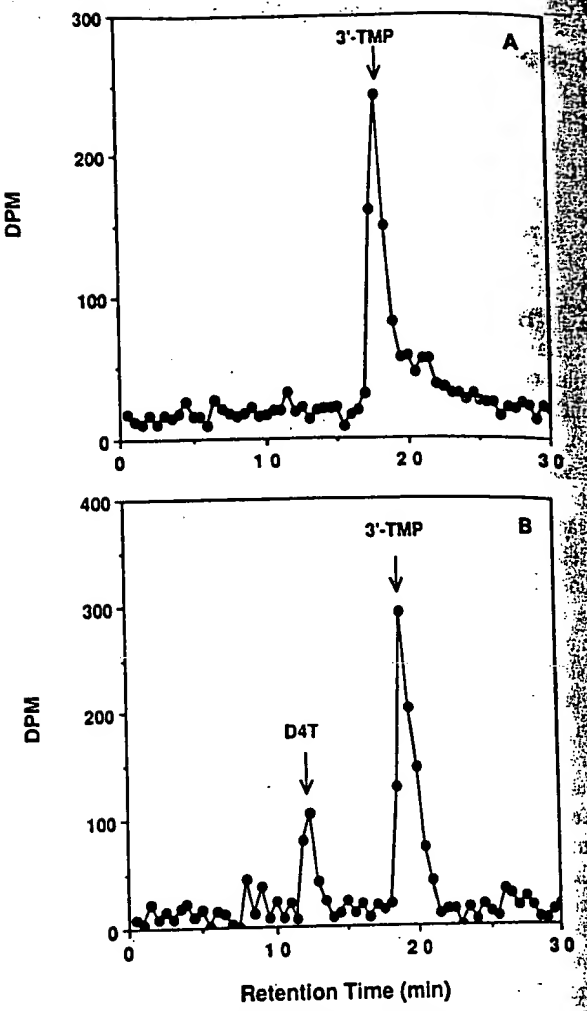


Fig. 5. Anion-exchange HPLC analysis of ^{3}H-labeled digested DNA isolated from cells exposed to either 10 μM [^{3}H]dThd (A) or 10 μM [^{3}H]D4T (B) for 24 hr. Purified DNA was digested by micrococcal nuclease and spleen 3′-phosphodiesterase.

Chemical stability of D4T-TP and ^{3}H-labeled D4T in cellular DNA. When D4T-TP, at a final concentration of 500 μM, was incubated at 37° and neutral pH in either McCoy's 5A nutrient medium or phosphate-buffered saline solution, a time-dependent degradation was observed, with approximately 60% of the initial concentration remaining after 48 hr of incubation (Fig. 7). Because this chemical degradation of D4T-TP was in a similar range, compared with the extent of D4T removal from DNA, as described above, it was important to determine the stability of D4T in newly synthesized DNA. After incubation of cells with 10 μM [^{3}H]D4T for 24 hr, DNA was purified and incubated at 37°, in McCoy's 5A nutrient medium, for time periods between 6 and 48 hr. At the specified times, aliquots were removed and spotted on disk filters, and radioactivity was determined after treatment with TCA and methanol. Of importance, the amount of radioactivity in DNA was not substantially altered over the 48-hr time of incubation, demonstrating that, once incorporated into cellular DNA, D4T as the D[illegible]MP form is chemically stable. Therefore, enzymatic excision, rather than chemical instability, of D4T from DNA of human BMC is a more likely mechanism for the observed removal of D4T from newly synthesized DNA.

Fig. 6. [illegible] of cells [illegible] AZT (● Values [illegible] from dif

Fig. 7. [illegible] and pH [illegible] from hu[illegible] tration [illegible] remainin[illegible] Mean ± [illegible]

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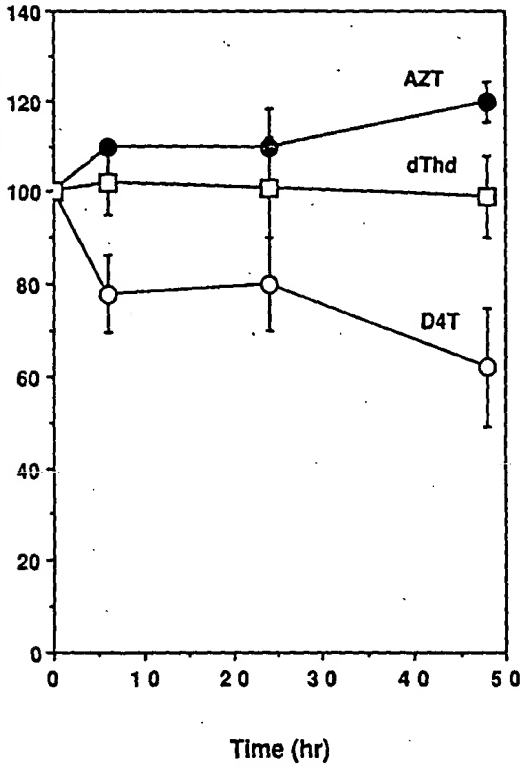
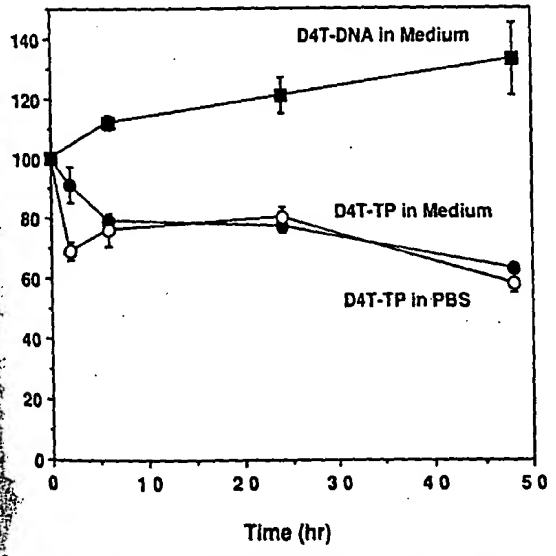


Fig. 6. ^{3}H removal from DNA over a 48-hr chase after a 24-hr exposure of cells to either 10 μM [^{3}H]D4T (○), 10 μM [^{3}H]dThd (□), or 10 μM [^{3}H]AZT (●). DNA was purified as described in Materials and Methods. Values are the mean ± standard error of three experiments, with cells from different donors.



Chemical stability in McCoy's 5A nutrient medium or PBS, at 37° H 7.4, of D4T-TP and [^{3}H]D4T in newly synthesized DNA isolated uman BMC exposed to 10 μM [^{3}H]D4T for 24 hr. D4T-TP concen- was determined by anion exchange HPLC, whereas the tritium ng in DNA was determined with a filter disk assay. Values are the standard error of three experiments.

Discussion

is a potent inhibitor of HIV replication, with activity rable to that of AZT in a large variety of cell lines (11), and yet is 20–100-fold less toxic than AZT in a human CFU-GM clonogenic assay (13, 20). This limited *in vitro* cytotoxicity against human BMC is consistent with the absence of major hematological toxicity in phase I clinical trials (15–17). In an attempt to elucidate the mechanism(s) of toxicity of 2′,3′-dideoxynucleosides for human BMC, it was of particular interest to study the cellular mechanism(s) that may be responsible for the decreased sensitivity of these human host cells to D4T. Previous studies have demonstrated that D4T has a different pattern of phosphorylation, compared with AZT, with dThd kinase being the rate-limiting step (8, 22, 24). In contrast, AZT-MP being poorly converted to the diphosphate is the rate-limiting step in AZT metabolism (23). Perturbation of intracellular deoxyribonucleoside 5′-triphosphate pools by AZT, through inhibition of thymidylate kinase by AZT-MP, had been suggested in early studies (8, 23), whereas D4T has been shown to exhibit little effect on the metabolism of exogenous labeled dThd and other nucleosides (8, 22, 24). Although this difference may have played a role in the decreased host toxicity of D4T, compared with AZT, more recent studies, including those from our group using human BMC, have ruled out depletion of dTTP by AZT as one mechanism responsible for AZT-induced bone marrow toxicity (4, 5, 7, 10, 25). Although the mechanism of anti-HIV activity of 2′,3′-dideoxynucleosides can be explained by their intracellular sequential phosphorylation by cellular kinases to their active 5′-triphosphate derivative (5), inconsistent data have been reported on the relationship between intracellular concentration of 5′-triphosphate metabolites of 2′,3′-dideoxynucleosides in host cells and their cellular toxicity (13, 35, 36). Table 1 demonstrates that D4T is also phosphorylated to its 5′-mono-, 5′-di-, and 5′-triphosphates, with a pattern similar to that reported in other cells (8, 22, 29, 30); of interest, intracellular D4T-TP values were about 2–3-fold higher, compared with AZT-TP levels, after incubation with similar extracellular concentrations of the parent drug (4). These data would, thus, further suggest that cellular toxicity cannot be correlated with the relative intracellular triphosphate levels. We, and others, have suggested that incorporation of AZT into nuclear DNA of host cells may be an important toxicity target (4, 25, 28), with DNA polymerase β being possibly responsible for that incorporation (26). In contrast, DNA polymerase α is probably not able to incorporate AZT-TP into elongating nuclear DNA, and the potential involvement of DNA polymerase δ is still debatable (25–27). In the present study, using cesium sulfate gradient centrifugation, D4T was detected in DNA of human BMC. But, of note, steady state levels of D4T incorporated into DNA were at least 10–50-fold lower than values measured after exposure of cells to AZT, under similar conditions. These data are, thus, consistent with the hypothesis that the extent of incorporation of 2′,3′-dideoxynucleosides into nuclear DNA correlates with bone marrow toxicity (4).

Because D4T-TP and AZT-TP do not exhibit major differences in affinity for human nuclear DNA polymerases (11), other mechanisms accounting for the substantial difference in incorporation into nuclear DNA between the two drugs were explored. Further analysis of radiolabeled DNA extracted from cells exposed to [^{3}H]D4T revealed that approximately 70% of the radioactivity was associated with dThd (Fig. 4). Formation of dThd was demonstrated to result from intracellular conversion of D4T to dThd, possibly by dThd phos-

quently, dTTP metabolically formed from D4T via dThd is rapidly utilized for DNA synthesis, competing with D4T-TP for its incorporation into DNA. Consistent with these data, Huang *et al.* (37) recently reported, by using *in vitro* polymerization assays, that high molecular weight DNA was still synthesized by DNA polymerase α and ϵ in the presence of D4T-TP and dTTP at a ratio as high as 30 to 1. Although D4T is probably converted to dThd through dThd phosphorylase, it is important to note that dThd phosphorylase activity is minimal in highly purified human lymphocytes (38) and even absent or undetectable in human T lymphocytes (39), a cell population that is a major target for HIV *in vivo* (40).

Consequently, the toxicity and probably the anti-HIV activity of D4T are dependent on cellular dThd phosphorylase levels, and modulation of D4T degradation by that cellular enzyme may contribute to the observed increased selectivity, compared with AZT.

Recent studies by Cheng and co-workers (25) have identified an exonucleolytic enzyme in human K-562 erythroleukemia cells that is suggested not to be associated with DNA polymerase activity and can remove 3′-terminal AZT or dideoxycytidine residues from DNA. In our study, no excision of AZT from DNA was detected, whereas label from D4T was substantially removed (Fig. 6). Although the D4T-TP was chemically unstable (Fig. 7), D4T, as the polynucleotide monophosphate form, was protected against chemical hydrolytic degradation after its incorporation into DNA (Fig. 7). This observation has been reported with other nucleosides (41) and suggests that an enzymatic repair mechanism is responsible for D4T excision from DNA. The lack of AZT excision from DNA in our study is inconsistent with the recently published data (25). However, a difference in our study is that nonradiolabeled compounds were added in the chase portion of the experiment. The rationale was to dilute the ^{3}H-labeled triphosphate pool (which declines slowly during that phase), thereby preventing further incorporation into DNA. A possible explanation for the absence of excision of AZT from DNA during the chase is that the exonuclease activity may be inhibited by AZT nucleotides. The inhibitory effects of high concentrations of nucleoside 5′-monophosphates on the 3′ to 5′ exonuclease activity are well recognized (42) and have been suggested to play a role in the mutagenic effects of 6-mercaptopurine, which, similar to AZT, mostly accumulates within cells as its 5′-monophosphate derivative. Preliminary studies[2] from our group have demonstrated the ability of AZT-MP, at concentrations of >100 μM, to inhibit the proofreading exonuclease activity associated with mammalian DNA polymerase δ, which may also provide a molecular mechanism for the carcinogenic effects observed with AZT. Although the effect of D4T-MP on 3′ to 5′ exonuclease activity has not yet been evaluated, cellular concentrations of D4T-MP are never high and are thus unlikely to have an influence on the excision process. Another, more speculative, possibility that could account for the different data between our study and that of Cheng's group (25) and the observed different patterns of excision from DNA of human BMC between D4T and AZT might be the presence of a specific repair enzyme. Differential expression in human BMC, compared with K-562 cells, and differential recognition of 2′,3′-dideoxynucleosides would also explain the observations.

[2] E. G. Bridges and J. P. Sommadossi, unpublished data.

In summary, decreased D4T incorporation into DNA of human BMC is probably a major mechanism for its low hematological toxicity, compared with AZT. The role of degradative host enzymes, including dThd phosphorylase, and repair enzymes in the minimal steady state incorporation of D4T into DNA further emphasizes the importance of cellular function, in addition to kinases, in modulating the anti-HIV activity of 2′,3′-dideoxynucleosides.

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References

1. Hirsh, M. S. Azidothymidine. *J. Infect. Dis.* **157**:427–431 (1988).
2. Richman, D. D., M. A. Fischl, M. H. Grieco, M. S. Gottlieb, P. A. Volberding, O. L. Laskin, J. M. Leedom, J. E. Groopman, D. Mildvan, M. S. Hirsch, G. G. Jackson, D. T. Durack, S. Nusinoff-Lehrman, and AZT Collaborative Working Group. The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex: a double-blind, placebo-controlled trial. *N. Engl. J. Med.* **317**:192–197 (1987).
3. Surbone, A., R. Yarchoan, N. McAtee, M. R. Blum, M. Maha, J. Allain, R. V. Thomas, H. Mitsuya, S. N. Lehrman, H. Kessler, C. E. Myers, and S. Broder. Treatment of the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex with a regimen of 3′-azido-2′,3′-dideoxythymidine (azidothymidine or zidovudine) and acyclovir. A pilot study. *Ann. Intern. Med.* **108**:534–540 (1988).
4. Sommadossi, J. P., R. Carlisle, and Z. Zhu. Cellular pharmacology of 3′-azido-3′-deoxythymidine, with evidence of incorporation into DNA of human bone marrow cells. *Mol. Pharmacol.* **36**:9–14 (1989).
5. Hao, Z., D. A. Cooney, N. R. Hartman, C. F. Perno, A. Fridland, A. L. De Vico, M. G. Sarngadharan, S. Broder, and D. G. Johns. Factors determining the activity of 2′,3′-dideoxy-nucleosides in suppressing human immunodeficiency virus *in vitro*. *Mol. Pharmacol.* **34**:431–435 (1988).
6. Harrington, J. A., W. H. Miller, and T. Spector. Effector studies of 3′-azidothymidine nucleotides with human ribonucleotide reductase. *Biochem. Pharmacol.* **36**:3757–3761 (1987).
7. Frick, L. W., D. J. Nelson, M. H. St. Clair, P. A. Furman, and T. A. Krenitsky. Effects of 3′-azido-3′-deoxythymidine on the deoxynucleotide triphosphate pools of cultured human cells. *Biochem. Biophys. Res. Commun.* **154**:124–129 (1988).
8. Balzarini, J., P. Herdewijn, and E. De Clercq. Differential patterns of intracellular metabolism of 2′,3′-didehydro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. *J. Biol. Chem.* **264**:6127–6133 (1989).
9. Balzarini, J., R. Pauwels, M. Baba, P. Herdewijn, E. De Clercq, S. Broder, and D. G. Johns. The *in vitro* and *in vivo* anti-retrovirus activity and intracellular metabolism of 3′-azido-2′,3′-dideoxythymidine and 2′,3′-dideoxycytidine are highly dependent on the cell species. *Biochem. Pharmacol.* **37**:897–903 (1988).
10. Fridland, A., M. C. Connelly, and R. Ashmun. Relationship of deoxynucleotide changes to inhibition of DNA synthesis induced by the antiretroviral agent 3′-azido-3′-deoxythymidine and release of its monophosphate by human lymphoid cells (CCRF-CEM). *Mol. Pharmacol.* **37**:665–670 (1990).
11. Hitchcock, M. J. M. 2′,3′-Didehydro-2′,3′-dideoxythymidine, an anti-HIV agent. *Antivir. Chem. Chemother.*, in press.
12. Mansuri, M. M., J. E. Starrett, I. Ghazzouli, M. J. M. Hitchcock, R. Z. Sterzycki, V. Brankovan, T. S. Lin, E. M. August, W. H. Prusoff, J. P. Sommadossi, and J. C. Martin. 1-(2,3-Dideoxy-β-D-glycero-pent-2-enofuranosyl)thymine (D4T): a highly potent and selective anti-HIV agent. *J. Med. Chem.* **32**:461–466 (1989).
13. Sommadossi, J. P., Z. Zhu, R. Carlisle, M.-Y. Xie, and D. A. Weidner. Pharmacologic studies of nucleosides active against the human immunodeficiency virus. *Ann. N. Y. Acad. Sci.* **616**:356–366 (1990).
14. Mansuri, M. M. M. J. M. Hitchcock, R. A. Buroker, C. L. Bregman, I. Ghazzouli, J. V. Desiderio, J. E. Starrett, R. Z. Sterzycki, and J. C. Martin. Comparison of biological properties *in vitro* and toxicity *in vivo* of thymidine analogues (D4T, FddT and AZT) active against HIV. *Antimicrob. Agents Chemother.* **34**:637–641 (1990).
15. Brown, M. J., and the Brown University AIDS Program, Clinical Trials Group. Phase I study of 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T) in patients with AIDS or ARC, in *VI International Conference on AIDS*, Abstract S.B.456, 200 (1990).
16. Dunkle, L., A. Cross, R. Gugliotti, R. Martin, M. Brown, and H. Mansuri. Dose-escalating study of safety and efficacy of dideoxy-didehydrothymidine (D4T) for HIV infection. *Antivir. Res.* **1**(suppl.):116 (1990).
17. Squires, K. E., W. Weiss, H. Sacks, J. Hassett, R. Gugliotti, and H. Mansuri. Effect of 2′,3′-didehydro-3′-deoxythymidine (D4T) on P24 antigenemia in patients with AIDS or ARC, in *VI International Conference on AIDS*, Abstract Th.A.241, 180 (1990).
18. Chen, C.-H., and Y.-C. Cheng. Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency

virus compound 2′,3′-dideoxycytidine. *J. Biol. Chem.* 264:11934–11937 (1989).
19. Cheng, Y.-C., C.-H. Chen, M. Vazquez-Padua, and C.-H. Tsai. Mechanism of delayed toxicity caused by anti-HIV nucleoside analogs, in *Advances in Chemotherapy of AIDS II* (R. B. Diasio and J. P. Sommadossi, eds.). Pergamon Press, Inc., New York, in press (1991).
20. Sommadossi, J. P., Z. Zhu, R. Carlisle, M.-Y. Xie, D. A. Weidner, and M. H. el Kouni. Novel pharmacological approaches to the treatment of AIDS and potential use of uridine phosphorylase inhibitors, in *Advances in Chemotherapy of AIDS* (R. B. Diasio and J. P. Sommadossi, eds.). Pergamon Press, Inc., New York, 63–73 (1990).
21. Cretton, E. M., M.-Y. Xie, R. J. Bevan, N. M. Goudgaon, R. F. Schinazi, and J. P. Sommadossi. Catabolism of 3′-azido-3′-deoxythymidine in hepatocytes and liver microsomes, with evidence of formation of 3′-amino-3′-deoxythymidine, a highly toxic catabolite for human bone marrow cells. *Mol. Pharmacol.* 39:258–266 (1991).
22. Ho, H.-T., and M. J. M. Hitchcock. Cellular pharmacology of 2′,3′-dideoxy-2′,3′-didehydrothymidine, a nucleoside analog active against human immunodeficiency virus. *Antimicrob. Agents Chemother.* 33:844–849 (1989).
23. Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weindhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* 83:8333–8337 (1986).
24. Marongiu, M. E., E. M. August, and W. H. Prusoff. Effect of 3′-deoxythymidin-2′-ene (D4T) on nucleoside metabolism in H9 cells. *Biochem. Pharmacol.* 39:1523–1528 (1990).
25. Vazquez-Padua, M. A., M. C. Starnes, and Y. C. Cheng. Incorporation of 3′-azido-3′-deoxythymidine into cellular DNA and its removal in a human leukemic cell line. *Cancer Commun.* 2:55–62 (1990).
26. Parker, W. B., E. L. White, S. C. Shaddix, L. J. Ross, R. W. Buckheit, Jr., J. M. Germany, J. A. Secrist III, R. Vince, and W. M. Shannon. Mechanism of inhibition of human immunodeficiency virus type I reverse transcriptase and human DNA polymerases α, β, and γ by the 5′-triphosphates of Carbovir, 3′-azido-3′-deoxythymidine, 2′,3′-dideoxyguanosine, and 3′-deoxythymidine. *J. Biol. Chem.* 266:1754–1762 (1991).
27. Huang, P., D. Farquhar, and W. Plunkett. Selective action of 3′-azido-3′-deoxythymidine 5′-triphosphate on viral reverse transcriptases and human DNA polymerases. *J. Biol. Chem.* 265:11914–11918 (1990).
28. Avramis, V. I., W. Markson, R. L. Jackson, and E. Gomperts. Biochemical pharmacology of zidovudine in human T-lymphoblastoid cells (CEM). *AIDS* 3:417–422 (1989).
29. Zhu, Z., H.-T. Ho, M.J.M. Hitchcock, and J. P. Sommadossi. Cellular pharmacology of 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T) in peripheral blood mononuclear cells. *Biochem. Pharmacol.* 39:R15–R19 (1990).
30. August, E. M., M. E. Marongiu, T.-S. Lin, and W. H. Prusoff. Initial studies on the cellular pharmacology of 3′-deoxythymidin-2′-ene (D4T): a potent and selective inhibitor of human immunodeficiency virus. *Biochem. Pharmacol.* 37:4419–4422 (1988).
31. Labarca, C., and K. Paigen. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102:344–352 (1980).
32. Pérignon, J. L., D. M. Bories, A. M. Houllier, F. Thuillier, and P. H. Cartier. Metabolism of pyrimidine bases and nucleosides by pyrimidine-nucleoside phosphorylases in cultured human lymphoid cells. *Biochim. Biophys. Acta* 928:130–136 (1987).
33. Friedkin, M., and D. W. Roberts. The enzymatic synthesis of nucleosides. I. Thymidine phosphorylase in mammalian tissue. *J. Biol. Chem.* 207:245–256 (1954).
34. Marsh, J. C., and S. Perry. Thymidine catabolism by normal and leukemic human leukocytes. *J. Clin. Invest.* 43:267–278 (1964).
35. Blakely, R. L., F. C. Harwood, and K. D. Huff. Cytostatic effects of 2′,3′-dideoxyribonucleosides on transformed human hemopoietic cell lines. *Mol. Pharmacol.* 37:328–332 (1990).
36. Hao, Z., D. A. Cooney, D. Farqhuar, C. F. Perno, K. Zhang, R. Masood, Y. Wilson, N. R. Hartman, J. Balzarini, and D. G. Johns. Potent DNA chain termination activity and selective inhibition of human immunodeficiency virus reverse transcriptase by 2′,3′-dideoxyuridine-5′-triphosphate. *Mol. Pharmacol.* 37:157–163 (1990).
37. Huang, P., D. Farquhar, and W. Plunkett. Selective action of 2′,3′-didehydro-2′,3′-dideoxythymidine triphosphate on HIV reverse transcriptase and human DNA polymerases. *Proc. Am. Assoc. Cancer Res.* 32:398 (1991).
38. Bodycotte, J., and S. Wolff. Metabolic breakdown of [^{3}H]thymidine and the inability to measure human lymphocyte proliferation by incorporation of radioactivity. *Proc. Natl. Acad. Sci. USA* 83:4749–4753 (1986).
39. Fox, R. M., S. K. Piddington, and A. A. Piper. Thymidine phosphorylase deficiency in cultured human T-leukemic lymphocytes: determinant of sensitivity to pyrimidine antimetabolites. *Proc. Am. Assoc. Cancer Res.* 20:262 (1979).
40. Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nygerye, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. Isolation of a T-lymphocytic retrovirus from a patient for acquired immune deficiency syndrome (AIDS). *Science (Washington D.C.)* 220:868–871 (1983).
41. Townsend, A. J., and Y. C. Cheng. Sequence-specific effects of ara-5-aza-CTP and ara-CTP on DNA synthesis by purified human DNA polymerases *in vitro:* visualization of chain elongation on a defined template. *Mol. Pharmacol.* 32:330–339 (1987).
42. Byrnes, J. J., K. M. Downey, B. G. Que, M. Y. W. Lee, V. L. Black, and A. G. So. Selective inhibition of the 3′ to 5′ exonuclease activity associated with DNA polymerases: a mechanism of mutagenesis. *Biochemistry* 16:3740–3746 (1977).

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